PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	Confirmation No. 4572
	)	
Feng Xu	)	Examiner: Michael C. Wilson
	)	
Serial No. 10/567,940	)	Group Art Unit: 1632
	)	
Filed: September 27, 2006	)	Atty. Dkt. No.: PP019817.0003

For: **INACTIVATED HOST CELL DELIVERY OF POLYNUCLEOTIDES ENCODING IMMUNOGENS**

BRIEF ON APPEAL

U.S. Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, VA 22314

Sir:

Appellant filed a Notice of Appeal on December 21, 2010. A panel decision on a Pre-Appeal Brief Request for Review was mailed February 16, 2011. Charge all required fees, including extension fees, to our Deposit Account No. 19-0733.

TABLE OF CONTENTS

I. REAL PARTY IN INTEREST

The real party in interest is Novartis Vaccines and Diagnostics, Inc.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF CLAIMS

Claims 1-13, 25, 27, 36, 38, 45-48 are pending and stand rejected. Claims 26, 28, 29, 37, 39, and 40 are withdrawn. Claims 14-24, 30-35, and 41-44 are canceled. Appellant appeals the rejection of claims 1-13, 25, 27, 36, 38, 45-48.

IV. STATUS OF AMENDMENTS

No amendment was filed after the final rejection.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Independent claim 1 is directed to a method for *in vivo* expression of an immunogen. Page 3, lines 6-8. The method comprises administering a bacterial host cell to a mammal. Page 3, lines 5-8; page 4, line 28 to page 5, line 2. The host cell comprises a polynucleotide that encodes an immunogen. *Id.* The polynucleotide is within the host cell genome, within a plasmid, or within a replicon. Page 4, line 30 to page 5 line 2. The host cell is unable to its own machinery to express the encoded immunogen. Page 6, lines 15-17. The immunogen is expressed *in vivo* by cells of the mammal. Page 2, lines 7-8.

Independent claim 8 is directed to a method of generating an immune response in a mammal. Page 2, lines 17-20. The method comprises administering a bacterial host cell to the mammal. *Id.*; page 4, line 28 to page 5, line 2. The host cell comprises a polynucleotide that encodes an immunogen. Page 4, line 28 to page 5, line 2. The host cell is unable to use its own machinery to express the encoded immunogen. *Id.*; page 6, lines 15-17. The immunogen is expressed *in vivo* by cells of the mammal, generating an immune response in the mammal against the immunogen. Page 2, lines 7-8 and 16-17.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Whether claims 1-13, 25, 27, 36, 38, and 45-48 are enabled under 35 U.S.C. § 112 ¶ 1.
2. Whether claims 1, 2, 5-8, 9, 12, 13, 25, 27, 36, 38, and 45-48 are *prima facie* obvious under 35 U.S.C. § 103(a).
3. Whether claims 1-13, 25, 27, 36, 38, and 45-46 are *prima facie* obvious under 35 U.S.C. § 103(a).

VII. ARGUMENT

A. Rejection Under 35 U.S.C. § 112 ¶1 for Lack of Enablement

1. Legal Standards

Section 112 ¶ 1 of 35 U.S.C. states that a patent specification must teach a person skilled in the relevant art how to make and use the invention claimed. The Examiner has the initial burden to establish a reasonable basis to question the enablement provided in the specification. *In re Wright*, 999 F.2d 1557, 1562 (Fed. Cir. 1993). The Examiner must not only explain why he doubts the statements in the specification's supporting disclosure, but also must support his assertions "with acceptable evidence or reasoning which is inconsistent with the contested statement." *In re Marzocchi*, 439 F.2d 220, 224 (C.C.P.A. 1971).

The proper standard for determining whether a specification meets the enablement requirement is whether any experimentation which may be needed to make and use the claimed invention is undue or unreasonable. *In re Wands*, 858 F.2d 731, 736-37 (Fed. Cir. 1988). The specification is addressed to those skilled in the art and need not provide knowledge generally known by those skilled in the art. *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986); *Genentech Inc. v. Novo Nordisk A/S*, 42 U.S.P.Q.2d 1001, 1005 (Fed. Cir. 1997). *Wands* sets forth relevant underlying fact inquiries:

> (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.

858 F.2d at 737.

The legal test for whether a disclosure provides adequate enablement for a generic claim is that "the scope of the claims must bear a *reasonable correlation* to the scope of enablement

provided by the specification to persons of ordinary skill in the art." *In re Fisher*, 427 F.2d 833, 839, 166 (C.C.P.A. 1970) (emphasis added), *cited with approval in Amgen, Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 1212 (Fed. Cir. 1991).

Whether a specification enables a claimed invention is a question of law based on underlying factual findings. *In re Vaeck*, 947 F.2d 488, 495 (Fed. Cir. 1991).

2. The Examiner has not made a *prima facie* case that claims 1-13, 25, 27, 36, 38, 45-48 are not enabled.

Each of the appealed claims recites a step of administering a bacterial host cell to a mammal. Independent claims 1 and 8 recite that the bacterial host cell comprises a polynucleotide encoding an immunogen. Independent claim 1 further specifies that the polynucleotide is "within the host cell genome, within a plasmid, or within a replicon." The Examiner has examined the pending claims "as they relate to the species Shigella" and acknowledges that the specification enables "a Shigella bacterial host cell to a mammal, wherein said Shigella comprises a plasmid encoding an immunogen." Page 2 of the Final Office Action mailed June 23, 2010. Dependent claims 5, 12, and 46 are directed to methods in which a plasmid comprises the polynucleotide encoding the immunogen. For this reason alone the rejection should not apply to claims 5, 12, and 46.

The rejection centers on whether the specification enables host cells in which the polynucleotide encoding the immunogen is <u>other</u> than "within a plasmid." As stated above, the legal test for whether a disclosure provides adequate enablement for a generic claim is that "the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art." *In re Fisher*, 427 F.2d at 839. As set

forth below, the specification provides adequate enablement for a polynucleotide encoding an immunogen, and the Examiner has provided no reasonable basis to question this enablement.

Polynucleotides encoding immunogens are well known in the art, as is their introduction into bacterial host cells, and the specification provides substantial guidance to a skilled artisan regarding both polynucleotides and their use to express an immunogen. The specification explains that a polynucleotide encoding an immunogen can be from a variety of sources:

> The term polynucleotide, as known in the art, generally refers to a nucleic acid molecule. A polynucleotide can include both double- and single stranded sequences and refers to, but is not limited to, cDNA from viral, prokaryotic or eukaryotic mRNA, genomic RNA and DNA sequences from viral (e.g. RNA and DNA viruses and retroviruses) or prokaryotic DNA, and especially synthetic DNA sequences.

Specification, page 4, lines 7-11. The specification further discloses that "the term [polynucleotide] also captures sequences that include any of the known base analogs of DNA and RNA and includes modifications such as deletions, additions and substitutions (generally conservative in nature) to the native sequence, so long as the nucleic acid molecule encodes a therapeutic or antigenic protein." *Id.*, lines 11-15. The specification further teaches that "a polynucleotide can include as little as 10 consecutive nucleotides, e.g., where the polynucleotide encodes an antigen." *Id.*, lines 21-23.

The specification provides examples of control elements that can be used to regulate expression from the polynucleotide in the mammal at page 6, lines 9-13: "For example, the polynucleotide sequence encoding the immunogen may be cloned into *Sal1* and *EcoRI* restriction sites of the eukaryotic expression vector pCMVKm2. This vector contains a cytomegalovirus (CMV) immediate-early enhancer/promoter and a bGH terminator." See also page 2, lines 14-15 disclosing that "[s]uitable promoters and vectors may be chosen to direct the expression of the immunogen." Based on the specification's teachings and the accumulated knowledge in the field

of molecular biology, one skilled in the art could readily prepare a polynucleotide encoding an immunogen and introduce it into a bacterial host cell.

Artisans typically introduce polynucleotides into bacteria in plasmid form. However, the state of the art is such that bacteria can be introduced in a variety of ways. In some cases, the polynucleotide may be incorporated in the genome, in other cases, the polynucleotide may be in an RNA or DNA replicon. Linear forms are also known. See, for example, Girons (Tab 10) at page 1809, col. 1 ¶ 3 to page 1810, col. 1 ¶ 1. The skilled artisan therefore can select from a variety of well-known, long-practiced approaches to prepare bacteria containing polynucleotides.

Claims 6 and 13 recite that the polynucleotide is incorporated into the host genome, and claim 45 recites that the polynucleotide is integrated within the host cell genome. The specification teaches that "polynucleotides encoding potential immunogens have been incorporated into open reading frames within bacterial genomes." Page 1, lines 12-14. See also page 4, line 30 to page 5, line 2, which teaches that bacteria can include a polynucleotide encoding the immunogen integrated within its genome. Making bacteria containing exogenous polynucleotides incorporated or integrated into the bacterial host cell genome is old in the art. See, for example, U.S. Pat. No. 5,695,976, "Stable Integration of DNA in Bacterial Genomes," which issued in 1997 (Tab 9).

Claims 47 and 48 specifically recite a DNA replicon and an RNA replicon, respectively. Plasmids and replicons are highly similar; indeed, in most cases the terms are synonymous. As noted above, the Examiner acknowledges that plasmids are enabled. Replicons, which may be RNA or DNA, are nucleic acids having a single origin of replication. Plasmids with only one replicating genetic unit are replicons. However, plasmids can contain more than one replicating genetic unit; *i.e.*, more than one replicon. See, for example, Sambrook (Tab 8):

> Usually a plasmid will contain only one origin of replication together with its associated cis-acting control elements (the whole genetic unit being defined as a 'replicon'). Very rarely, however, plasmids that been generated by fusion will contain more than one replicon; in such cases, only one replicon is active.

Thus, because replicons propagate in bacteria, they can readily be maintained within bacteria using techniques well known in the art.

The Examiner contends that "the specification does not correlate the use of plasmids to using linear strands of non-plasmid DNA, to RNA, to replicons (DNA or RNA) or to any virus (including retrovirus, specifically HIV) as the polynucleotide." Office Action of June 23, 2010 at page 3. Thus, the Examiner concludes, "[i]t would have required ... undue experimentation to determine how to use any polynucleotide, specifically linear strands of non-plasmid DNA, RNA replicons (DNA or RNA) or any virus including retrovirus (especially HIV) to express immunogens in mammal as claimed." *Id.*[1]

Nothing in these statements supplies a reasonable basis required to establish a *prima facie* case of non-enablement. The Examiner simply asserts that the specification does not "correlate" plasmids to other polynucleotides then summarily concludes that undue experimentation would be required to use any polynucleotide. Final Office Action mailed June 23, 2010, at page 3. The Examiner has provided neither reasoning nor evidence to support the assertion that one of skill in the art could not use any polynucleotide. The Examiner has not provided an explanation why one

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[1] A particular focus of the rejection appears to be the use of an entire retroviral genome inside the bacterial host cell: "Applicants' argument is not persuasive because the polynucleotide can still be with the host cell genome which includes any nucleic acid sequence including a retrovirus." Office Action of June 23, 2010 at page 3. This objection is not well-founded. One of skill in the art would not move the entire retroviral genome *en mass* into a bacterial cell. Rather, a skilled artisan would identify the relevant polynucleotides encoding the desired immunogens and introduce them into the bacteria. Such an approach does not preclude introducing a polynucleotide encoding multiple immunogens. The immunogens encoded by the polynucleotides will be expressed in the mammal, provided that mammalian control elements can direct expression of the immunogen. Control elements to direct expression of polynucleotides in mammalian cells are well known.

of skill in the art could not take any polynucleotide encoding an immunogen and introduce it into a bacterial host cell, including within the host cell genome, within a plasmid, or within a replicon. Nor has the Examiner cited any scientific studies that support an assertion a skilled artisan could not make and use bacterial host cells comprising a polynucleotide. The Examiner therefore has not established that any of claims 1-13, 25, 27, 36, 38, 45-48 is not enabled.

3. The Specification Enables use of Bacterial Host Cells Other than *Shigella*.

Finally, as noted above, the claims have been examined "as they relate to the species Shigella." The specification teaches use of *Shigella flexneri* on page 5, lines 6-8, and in the working Example on pages 18-19. Claims 27 and 38 recite that the bacterial host cell is *Shigella flexneri* and should be allowed on this basis alone. For the sake of completeness, Appellant notes that the arguments set forth above apply with equal force to other species of bacterial host cells.

The specification teaches that "[b]acterial host cells suitable for use in the invention include *E. coli*, *Shigella spp.*, *Bordella spp.*, *Salmonella spp.*, *Bacillus spp.*, *Streptococcus spp.*, *Mycobacteria spp.* or other bacterial species, or other microorganisms which can harbor plasmids or DNA or RNA replicons." Page 5, lines 3-6. The specification also teaches various methods of rendering a bacterial host cell "unable to use its own machinery to express the encoded immunogen." Heat treatment, as recited in claims 2 and 9, is taught on pages 18-19. UV light exposure (as recited in claims 3 and 10) and hydrogen peroxide treatment (as recited in claims 4 and 11) are taught on page 6, lines 15-19. The skilled artisan could readily apply any of these methods to bacterial host cells other than *Shigella*, including those explicitly taught in the specification and recited in claims 25 and 36 (*E. coli*, *Shigella spp.*, *Bordella spp.*, *Salmonella spp.*, *Bacillus spp.*, *Streptococcus spp.*, *Mycobacteria spp.*).

4. Conclusion

The determination of enablement should be based on the weight of all the evidence of record. *Wands*, 858 F.2d at 737; M.P.E.P. § 2164.04. The specification provides ample guidance to make a bacterial host cell comprising a polynucleotide encoding an immunogen and to treat the bacteria such that they are unable to use their own machinery to express the immunogen. The molecular biology approaches required to prepare a polynucleotide that can express an immunogen in a mammal and introduce it to bacteria are well-known, and the underlying biology required to express a protein in a mammalian cell is a predictable art. One of skill in the art could readily prepare a bacterial host cell comprising a polynucleotide encoding an immunogen.

Together with the maturity of the molecular biology arts and the predictability of protein expression in mammalian cells, the facts set forth above compel the legal conclusion that the specification enables the full scope of claims 1-13, 25, 27, 36 38, 45-48. The Examiner has provided no evidence to the contrary. The rejection under 35 U.S.C. § 112 ¶ 1 should therefore be reversed.

B. Rejection under 35 U.S.C. § 103(a) over Xu, zur Megede, Masschalck, and Raettig

The issue in this rejection is whether the Examiner has established that claims 1, 2, 5-8, 9, 12, 13, 25, 27, 36, 38, and 45-48 are *prima facie* obvious. Appellants understand, pursuant to a teleconference with Examiner Wilson, that this rejection would be withdrawn in view of the arguments made in the pre-appeal brief request for review. As a result of the procedural posture of the case, however, Appellants have responded to the outstanding rejection in full.

1. Principles of Law

A claimed invention is not patentable if the subject matter of the claimed invention would have been obvious to a person having ordinary skill in the art. 35 U.S.C. § 103(a). Facts relevant to a determination of obviousness include (1) the scope and content of the prior art, (2) any differences between the claimed invention and the prior art, (3) the level of ordinary skill in the art, and (4) relevant objective evidence of obviousness or non-obviousness. *Graham v. John Deere Co. of Kansas City*, 383 U.S. 1, 17-18 (1966). "[O]bviousness requires a suggestion of all limitations in a claim." *CMFT, Inc. v. Yieldup Intern. Corp.*, 349 F.3d 1333, 1342 (Fed. Cir. 2001) (citing *In re Royka*, 490 F.2d 981, 985 (C.C.P.A. 1974)).

The determination of whether obviousness is established by combining references must be based on "what the combined teachings of the references would have suggested to those of ordinary skill in the art." *In re GPAC Inc.*, 57 F.3d 1573, 1581 (Fed. Cir. 1995) (internal quotations omitted). In *KSR Int'l Co. v. Teleflex Inc.*, the Supreme Court emphasized "an expansive and flexible approach" to the obviousness question. 550 U.S. 389 at 418 (2007). The Court also reaffirmed, however, that "a patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art." *Id.* The Court stated:

> [I]t can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements *in the way the claimed new invention does* . . . because inventions in most, if not all, instances rely upon building blocks long since uncovered, and claimed discoveries almost of necessity will be combinations of what, in some sense, is already known.

550 U.S. at 418-19 (emphasis added). *See also Id.* at 418 (requiring a determination of "whether there was an apparent reason to combine the known elements *in the fashion claimed* by the patent at issue") (emphasis added).

2. Findings of Fact

The Graham factors continue to define the inquiry whether an invention is *prima facie* obvious. KSR, 550 U.S. at 407. This inquiry reveals the following relevant facts.

a. Scope and Content of Prior Art

The Examiner cites a proposed combination of four references: Xu (Tab 2), zur Megede (Tab 3), Masschalck (Tab 4), and Raettig (Tab 5) as rendering claims 1, 2, 5-8, 9, 12, 13, 25, 27, 36, 38, and 45-48 *prima facie* obvious. Xu is the primary reference. The Examiner relies on Xu to teach attenuated *Shigella* comprising a plasmid encoding HIV-1 SF2 Gag, under the control of a mammalian promoter, to mice. Office Action of June 23, 2010 at page 4. Xu, however, cannot be used in a rejection against the pending claims because a declaration under 37 C.F.R § 1.131 of the sole inventor, Dr. Feng Xu (Tab 1), establishes that Xu is not prior art to the present application.

An effective declaration under 37 C.F.R § 1.131 requires a showing of facts that establishes reduction to practice prior to the effective date of the reference. 37 C.F.R § 1.131. The declaration need not show the identical disclosure of the reference. Rather,

> If the affidavit contains facts showing a completion of the invention commensurate with the extent of the invention as claimed is shown in the reference or activity, the affidavit or

> declaration is sufficient, whether or not it is a showing of the identical disclosure of the reference or the identical subject matter involved in the activity.

M.P.E.P § 715.02 (8th ed., Sept. 2007) (citing *In re Wakefield*, 422 F.2d 897, 964 (C.C.P.A. 1970).

Xu teaches using attenuated bacteria containing plasmids encoding an antigen and administering the bacteria to mice to induce an immune response. Dr. Xu's declaration provides the required showing to antedate Xu. First, Dr. Xu states all work described in his declaration was performed before October 25, 2002, the date that Xu was publicly available on the internet. Declaration at ¶ 2. Second, Dr. Xu's declaration explains that he prepared heat-killed bacteria containing a plasmid that encodes an antigen, administered the bacteria to mice, and induced an immune response. Dr. Xu states that he immunized mice with killed *Shigella flexneri* carrying a plasmid containing DNA encoding an HIV gag antigen. Declaration at ¶¶ 3, 5. Dr. Xu states that the "[b]acterial cells were heat killed." Declaration at ¶ 5. Dr. Xu states that the data demonstrated that immunization of mice with killed bacterial cells which harbor a plasmid that contains DNA encoding HIV gag causes an immune response directed against the HIV gag. *Id.* As noted above, all of this work was performed before October 25, 2002.

Dr. Xu's declaration contains facts sufficient to show that he prepared heat-killed bacteria containing a plasmid encoding an antigen, administered the bacteria to mice, and induced an immune response before Xu's publication date. Dr. Xu's declaration meets the requirements of 37 C.F.R. § 1.131 and is therefore sufficient to antedate Xu.

With Xu removed as a reference, the rejection relies only on zur Megede, Masschalck, and Raettig. Zur Megede teaches using modified HIV gene sequences in plasmids for improved expression and the use of CMV promoters in the plasmids. Page 2629, col. 1, ¶ 2. The plasmids

are administered to mammals but are not contained within bacteria. Masschalck teaches inactivating gram-negative bacteria, including *Shigella*, but for food preservation applications. Page 339, col. 1, ¶ 1. Masschalck teaches inactivating bacteria using high pressure and lysozyme, an enzyme that degrades bacterial cell walls. Page 339, Abstract. Raettig, which is only a short abstract, teaches preparing a vaccine against *Shigella*, and other bacterial species, by heat-inactivating the bacteria and administering the heat-inactivated bacteria to mice.

b. Difference Between the Claimed Invention and the Prior Art

The second factual inquiry under *Graham* is to ascertain the differences between the claimed invention and the cited art. 383 U.S. at 17. In determining these differences, the question is not whether the differences themselves would have been obvious, but whether the claimed invention as a whole would have been obvious. *Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 1538 (Fed. Cir. 1983). Moreover, "[a]ll words in a claim must be considered in judging the patentability of that claim against the prior art." *In re Wilson*, 424 F.2d 1382, 1383 (C.C.P.A. 1970).

Each of the appealed claims includes a step of "administering a bacterial host cell to a mammal, wherein said host cell comprises a polynucleotide encoding an immunogen, wherein the host cell is unable to use its own machinery to express the encoded immunogen, and wherein the immunogen is expressed *in vivo* by the cells of the mammal."

The references do not teach or suggest this feature. Zur Megede teaches modified HIV gene sequences in plasmids for improved expression. The plasmids are administered to mammals. Zur Megede does not teach or suggest administering plasmids inside bacteria to mammals. Masschalck teaches inactivating gram-negative bacteria, including *Shigella*, for food pasteurization. Page 339, col. 1, ¶1. Masschalck inactivates bacteria using high pressure and

lysozyme, an enzyme that degrades bacterial cell walls. Page 339, Abstract. Masschalck's teachings are unrelated to vaccine applications. The Raettig abstract teaches heat-inactivation of *Shigella* and other heat-inactivated bacteria and administration of the heat-inactivated bacteria to mice as a vaccine against the bacteria. Nothing in Raettig suggests modifying the bacteria to include a polynucleotide encoding an immunogen that can be expressed by the cells of the mammal.

3. Analysis

Xu, the primary reference, is not prior art to the pending application. Nothing in the disclosures of the remaining references—zur Megede, Masschalck, and Raettig—would have provided one of ordinary skill with any motivation to make a bacterial host cell comprising polynucleotides encoding immunogens, wherein the bacterial host cell is unable to use its own machinery to express the encoded immunogen. First, Masschalck is directed to developing pasteurization technology for food preparation and is silent regarding genetically-modified bacteria and their use. Masschalck does not teach or suggest anything about inducing immune responses or about expressing immunogens in mammals. One of ordinary skill would therefore not even consider combining Masschalck with zur Megede or Raettig.

Raettig is directed to producing a whole-cell vaccine against bacteria. In contrast, zur Megede is directed to producing immune responses against an encoded immunogen. There is no suggestion in Raettif or zur Megede to express an immunogen in mammalian cells by administering a bacterial host cell comprising a polynucleotide encoding the immunogen, wherein the host cell is unable to use its own machinery to express the immunogen.

Nothing in the cited references, alone or in combination, would have provided the ordinary artisan with any reason to administer to a mammal a bacterial host cell comprising a

polynucleotide that encodes an immunogen, wherein the host cell is unable to use its own machinery to express the immunogen, and wherein the immunogen is expressed *in vivo* by cells of the mammal. There is no evidence of record to the contrary. The Examiner has therefore not established that claims 1, 2, 5-8, 9, 12, 13, 25, 27, 36, 38, and 45-48 are *prima facie* obvious. The rejection should be reversed.

C. Rejection Under 35 U.S.C. § 103(a) Over Xu, Zur Megede, Masschalck, Raettig, Chang, and Kruithof

The issue in this rejection is whether the Examiner has established that claims 1-13, 25, 27, 36, 38, and 45-46 are *prima facie* obvious.

The analysis above applies with equal force to the rejection of claims 1-13, 25, 27, 36, 38, and 45-46 over Xu, zur Megede, Masschalck, Raettig, Chang (Tab 6), and Kruithof (Tab 7).

As explained above, Xu is not prior art to the pending claims, and the teachings of zur Megede, Masschalck, and Raettig—even if, *arguendo*, combined—would not make claims 1, 2, 5-8, 9, 12, 13, 25, 27, 36, 38, and 45-48 *prima facie* obvious. Neither Chang nor Kruithof cures these deficiencies with respect to claims 1, 2, 5-8, 9, 12, 13, 25, 27, 36, 38, and 45-48 or makes claims 3, 4, 10, or 11 *prima facie* obvious.

Chang is cited only as teaching inactivating *Shigella* using UV light. Office Action of June 23, 2010 at page 6. Kruithof is cited only as teaching inactivating bacteria using UV light. *Id.* Nothing in Chang or Kruithof provides any reason to express an immunogen in a mammal by administering a bacterial host cell comprising a polynucleotide encoding the immunogen, wherein the bacterial cell cannot use its own machinery to express the encoded immunogen. Chang assessed survival of various microorganisms, including *Shigella*, after UV light exposure.

Chang is silent regarding genetically-modified bacteria and their use in expressing proteins in mammals or in inducing immune responses in mammals.

Kruithof is directed to disinfecting water contaminated by microorganisms. Kruithof teaches killing the microorganisms using hydrogen peroxide and UV light. Kruithof, like Chang, is silent regarding genetically-modified bacteria and their use in expressing proteins in mammals or in inducing immune responses in mammals. Neither reference provides any reason to prepare a bacterial host cell comprising a polynucleotide encoding an immunogen, wherein the host cell is unable to use its own machinery to express the encoded immunogen.

Thus, even if combined, nothing in the disclosures of zur Megede, Masschalck, Raettig, Chang, and Kruithof, alone or in combination, would have provided the ordinary artisan with any reason to administer to a mammal a bacterial host cell comprising a polynucleotide that encodes an immunogen, wherein the host cell is unable to use its own machinery to express the immunogen, and wherein the immunogen is expressed *in vivo* by cells of the mammal. There is no evidence of record to the contrary.

The Examiner has not established that claims 1-13, 25, 27, 36, 38, and 45-46 are *prima facie* obvious. The rejection of claims 1-13, 25, 27, 36, 38, and 45-46 as obvious over Xu, zur Megede Masschalck, Raettig, Chang, and Kruithof should therefore be reversed.

VIII. CONCLUSIONS

1. The Examiner has not established a *prima facie* case that claims 1-13, 25, 27, 36 38, 45-48 are not enabled, therefore, the rejection under 35 U.S.C. § 112 ¶1 should be reversed.

2. The Examiner has not established a *prima facie* case that claims 1, 2, 5-8, 9, 12, 13, 25, 27, 36, 38, and 45-48 are obvious over Xu, zur Megede, Masschalck, and Raettig; therefore, the rejection under 35 U.S.C. § 103(a) should be reversed.

3. The Examiner has not established a *prima facie* case that claims 1, 2, 5-8, 9, 12, 13, 25, 27, 36, 38, and 45-48 are obvious over Xu, zur Megede, Masschalck, Raettig, Chang, and Kruithof; therefore, the rejection under 35 U.S.C. § 103(a) should be reversed.

Respectfully submitted,

BANNER & WITCOFF, LTD.

Date: July 18, 2011

By: /Fraser D. Brown/
Fraser D. Brown
Registration No. L0617
Attorney for Applicant
1100 13th Street, N.W., Suite 1200
Washington, DC 20005-4051
(202) 824-3000 (telephone)
(202) 824-3001 (facsimile)

IX. CLAIMS APPENDIX

1. A method for *in vivo* expression of an immunogen comprising:

administering a bacterial host cell to a mammal, wherein said host cell comprises a polynucleotide encoding an immunogen, wherein the polynucleotide is within the host cell genome, within a plasmid, or within a replicon, wherein the bacterial host cell is unable to use its own machinery to express the encoded immunogen, and wherein the immunogen is expressed *in vivo* by cells of the mammal.

2. The method of claim 1 wherein the bacterial host cell is unable to use its own machinery to express the encoded immunogen due to heat treatment.

3. The method of claim 1 wherein the bacterial host cell is unable to use its own machinery to express the encoded immunogen due to ultra-violet light exposure.

4. The method of claim 1 wherein the bacterial host cell is unable to use its own machinery to express the encoded immunogen due to hydrogen peroxide treatment.

5. The method of claim 1 wherein a plasmid comprises the polynucleotide encoding the immunogen.

6. The method of claim 1 wherein the polynucleotide encoding the immunogen is incorporated into the host cell genome.

7. The method of claim 1 wherein the expressed immunogen generates an immune response in the mammal.

8. A method of generating an immune response in a mammal comprising:

administering a bacterial host cell to said mammal, wherein said host cell comprises a polynucleotide encoding an immunogen, wherein the host cell is unable to use its own machinery to express the encoded immunogen, wherein the immunogen is expressed *in vivo* by cells of the mammal, thereby generating an immune response in the mammal against the immunogen.

9. The method of claim 8 wherein the bacterial host cell is unable to use its own machinery to express the encoded immunogen due to heat treatment.

10. The method of claim 8 wherein the bacterial host cell is unable to use its own machinery to express the encoded immunogen due to ultraviolet light exposure.

11. The method of claim 8 wherein the bacterial host cell is unable to use its own machinery to express the encoded immunogen due to hydrogen peroxide treatment.

12. The method of claim 8 wherein a plasmid comprises the polynucleotide encoding the immunogen.

13. The method of claim 8 wherein the polynucleotide encoding the immunogen is incorporated into the host cell genome.

25. The method of claim 1 wherein the bacterial host cell is selected from the group consisting of *E. coli*, *Shigella spp*, *Bordella spp*, *Salmonella spp*, *Bacillus spp*, *Streptococcus spp*, and *Mycobacteria spp*.

27. The method of claim 25 wherein the bacterial host cell is *Shigella flexneri*.

36. The method of claim 8 wherein the bacterial host cell is selected from the group consisting of *E. coli*, *Shigella spp*, *Bordella spp*, *Salmonella spp*, *Bacillus spp*, *Streptococcus spp*, and *Mycobacteria spp*.

38. The method of claim 36 wherein the bacterial host cell is *Shigella flexneri*.

45. The method of claim 1 wherein the polynucleotide encoding an immunogen is integrated within the host cell genome.

46. The method of claim 1 wherein the polynucleotide encoding an immunogen is within a plasmid.

47. The method of claim 1 wherein the replicon is a DNA replicon.

48. The method of claim 1 wherein the replicon is an RNA replicon.

X. EVIDENCE APPENDIX

EVIDENCE	LOCATION IN THE RECORD	TAB
Declaration under 37 C.F.R. § 1.131 of Dr. Feng Xu	filed with a response on November 25, 2009.	1
Xu *et al.*, "Immunogenicity of an HIV-1 gag DNA vaccine carried by attenuated Shigella," Vaccine. 2003 Jan 30;21(7-8):644-8	IDS submitted March 19, 2008	2
Zur Megede *et al.*, "Increased expression and immunogenicity of sequence-modified human immunodeficiency virus type 1 gag gene," J Virol. 2000 Mar;74(6):2628-3	Cited in Office Action mailed June 26, 2009	3
Masschalck *et al.*, "Inactivation of gram-negative bacteria by lysozyme, denatured lysozyme, and lysozyme-derived peptides under high hydrostatic pressure," Appl Environ Microbiol. 2001 Jan;67(1):339-44	Cited in Office Action mailed June 26, 2009	4
Raettig, "An oral enteritis-vaccine composed of twelve heat-inactivated Enterobacteriaceae 3. Communication: studies on efficacy tests in mice protection tests," Zentralbl Bakteriol Mikrobiol Hyg A. 1981 Nov;250(4):511-20, abstract	Cited in Office Action mailed June 26, 2009	5
Chang *et al.*, "UV inactivation of pathogenic and indicator microorganisms," Appl Environ Microbiol. 1985 Jun;49(6):1361-5, abstract	Cited in Office Action mailed June 26, 2009	6
Kruithof *et al.*, "UV/H_2O_2-treatment: The ultimate solution for pesticide control and disinfection," Proceedings- Annual Conference, American Water Works assoc. 2000, p331-334, abstract	Cited in Office Action mailed June 26, 2009	7
Sambrook *et al.*, Molecular Cloning: a Laboratory Manual 2nd ed, 1989. Vol 1, Page 1.3	IDS submitted March 17, 2010	8
U.S. Pat. No. 5,695,976, "Stable Integration of DNA in Bacterial Genomes," issued December 9, 1997	IDS submitted March 17, 2010	9
Girons *et al.*, "Molecular biology of the Borrelia, bacteria with linear replicons," Microbiology. 1994 Aug;140 (Pt 8):1803-16	IDS submitted March 17, 2010	10

XI. RELATED PROCEEDINGS APPENDIX

NONE.

TAB 1

Declaration under 37 C.F.R. § 1.131 of Dr. Feng Xu

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Conf. No. 4572
Feng Xu	)	Group Art Unit 1632
Serial No: 10/567,940	)	Examiner: Michael C. Wilson
Filed: September 27, 2006	)	Atty. Docket No. PP019817.0003

For: **INACTIVATED HOST CELL DELIVERY OF POLYNUCLEOTIDES ENCODING IMMUNOGENS**

DECLARATION OF DR. FENG XU UNDER 37 C.F.R. § 131

U.S. Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, VA 22314

I, Feng Xu, hereby declare the following:

1. I am named as the sole inventor of the subject matter claimed in application Serial No. 10/567,940. At the time this application was filed, I was an employee of Chiron Corporation.

2. All work described in this declaration was performed in the United States before October 25, 2002.

3. Before October 25, 2002, I demonstrated that immunization of animals directly with killed bacterial cells of *E. coli* and *Shigella flexineri* with plasmid containing DNA encoding an antigen could generate immune responses directed against the antigen encoded by the DNA.

4. Results showing a cellular response (interferon-γ production) in mice after intramuscular immunization with killed recombinant bacterial cells carrying a plasmid containing DNA encoding HIV gag protein are shown in the table below.

Vaccine	Interferon-γ production (pg/10^6 spleen cells)
Killed *E. coli* DH5α (5×10^5 cfu)	96.4
Killed *Shigella* 1207-3 (5×10^5 cfu)	313
Killed *Shigella* 1207-3 (5×10^4 cfu)	175
Saline	0

5. The data in the table were generated as follows. Bacterial cells were heat killed and injected in a 2 x 50 µl volume into the tibialis anterior muscle of each mouse leg. Saline was used as a negative control. Spleen cells were collected after the immunized mice were challenged intra-peritoneally with a recombinant vaccinia virus that expresses HIV gag protein. The collected spleen cells were then stimulated with HIV gag p7 peptide to measure the cells' ability to respond to the immunogen by producing interferon-γ. The data demonstrate that immunization of mice with killed bacterial cells which harbor a plasmid that contains DNA encoding HIV gag causes an immune response directed against the HIV gag

6. All statements I made in this declaration of my own knowledge are true. I believe all statements made on information and belief to be true. I made these statements with the knowledge that willful false statements and the like so made are punishable by fine or

imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent.

Dated: 10/22/09

Feng Xu, Ph.D.

TAB 2

Xu *et al.*, "Immunogenicity of an HIV-1 gag DNA vaccine carried by attenuated Shigella," Vaccine. 2003 Jan 30; 21(7-8):644-8

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Vaccine 21 (2003) 644–648

Vaccine

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Immunogenicity of an HIV-1 *gag* DNA vaccine carried by attenuated *Shigella*

Fengfeng Xu, Mei Hong, Jeffrey B. Ulmer*

Vaccines Research, Chiron Corporation, 4560 Horton St., mail stop 4-3, Emeryville, CA 94608, USA

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Abstract

The use of live attenuated invasive bacteria as a carrier for DNA-based vaccines has been reported recently. In this study, we used a *Shigella flexneri* serotype 2a *rfbF* mutant for immunization of a DNA vaccine coding for HIV-1 SF2 Gag. The recombinant bacterial vector delivered *gag* DNA to mammalian cells in vitro resulting in Gag protein expression, and was found to have a low level of pathogenicity among a number of *Shigella* cell spread defective mutants tested. Intranasal immunization of mice with live recombinant bacterial cells induced a gag-specific cellular immune response similar to that seen with i.m. injection of naked DNA. Importantly, a strong boosting effect was observed in mice primed with DNA, suggesting utility of bacterial vectors in prime-boost vaccination regimens.
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Keywords: *Shigella*; DNA vaccine; HIV gag

1. Introduction

Gene transfer from bacteria to mammalian cells was first observed when tandem copies of the SV 40 virus genome carried by *Escherichia coli* was transferred into cocultured mammalian cells [1]. The transfer efficiency was very low, likely due to the poor frequency of bacteria entering mammalian cells and escaping from endosomes into the cytoplasm. An improved transfer system was later developed using invasive *Shigella* strains, which readily gain access to the cytoplasm of infected cells [2,3]. Such a vector system for DNA delivery has recently been used in prototype vaccines. Immunization with recombinant invasive bacteria including *Shigella*, *Salmonella* and *Listeria* carrying plasmid DNA vaccines has been shown to induce protective immune responses in mice (for reviews, see [4,5]). Using human enteric bacteria is particularly advantageous because of their ability to infect human colonic mucosa, and their tropism for and activation of dendritic cells and macrophage of internal mucosa. Thus, they are very efficient for delivery of DNA vaccines to antigen presenting cells (APCs) in the mucosa for induction of potent systemic and local immune responses. Such responses may be critical for the development of an effective prophylactic HIV vaccine, as a large number of HIV transmissions are through human mucosal routes (for reviews, see [6,7]). Recently, *Shigella* carrying DNA encoding HIV gp120 were shown to be immunogenic in mice [8,9]. Specifically, strains mutated in the *asd* (diaminopimelic acid biosynthesis), *aroA* (aromatic amino acid synthesis) and *icsA* (control of cell to cell spread) genes were used. We report here that an attenuated strain of *Shigella* mutated in the *rfbF* (O-antigen synthesis) gene and carrying a plasmid encoding HIV gag was effective for inducing local and systemic immune responses, and may be useful in prime-boost vaccination regimens with DNA vaccines.

2. Materials and methods

2.1. Bacterial strains and plasmids

Shigella flexneri serotype 2a wild-type host SA100 and its derived mutants with Tn5 transposon inserted in single genes of *vpsC*, *ispA*, *icsA*, *rfbF*, and *ipaC* [10,11], were obtained from University of Texas at Austin. Other SA100 derived *dapB* and *dsbA* mutants were made in this study by P1 phage transduction using donor hosts *S. flexneri* EB2104 (*dapB*, Tn5 insertion) [12] and *E. coli* JCB571 (*dsbA*, Tn5 insertion) [13], respectively. *Shigella* strains were cultured in Tryptic Soy medium and *E. coli* in LB. Diaminopimelic acid (Dap, 100 μg/ml) was added in the media for growing *dapB* mutants. Kanamycin (100 μg/ml) and ampicillin

* Corresponding author. Tel.: +1-510-923-5140; fax: +1-510-923-2586.
E-mail address: jeffrey_ulmer@chiron.com (J.B. Ulmer).

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(100 μg/ml) were used for selection of drug resistant bacteria. Except for the *ipaC* mutant, single red (a phenotype of invasiveness) colony of *Shigella* strains was isolated on Tryptic Soy/Congo red plates and propagated.

DNA vaccine pCMVH.gag, encoding the HIV-1 SF2 *gag* gene, was modified from plasmid pCMVgag [14] with the replacement of a new plasmid replication origin (*ori*) and ampicilin resistance gene (*amp*^{r}). The pCMVH.gag was found stable and replicating with a high copy number in *Shigella* (data not shown). The new *ori* region was made by PCR from pNOP6 [15] using primers 5′-TATAGCTAGCACGTAGAAAAGATCAAAGGATCTTC (underlined sequence: *Nhe*I) and 5′-TATAGGTACCGTAAAAAGGCCGCGTTGCTGGCG (underlined sequence: *Kpn*I); and the *amp*^{r} gene region is similarly made from pGEM3 (Promega) using primers 5′-TATACCCGGGAAATGTGCGCGGAACCCCTATTTG (underlined sequence: *Xma*I) and 5′-TATAGGTACCAATCTAAAGTATATATGAGTAAACTTGG (underlined sequence: *Kpn*I). Construction of pCMVH.gag was made in two steps. The first step was the construction of pCMVH.gag-Km by ligation of the new *ori* region with the *ori*-deleted pCMVgag PCR fragment made using primers 5′-TATAGCTAGCGGCCGCGGAATTTCGACTCTAGG (underlined sequence: *Nhe*I) and 5′-TATAGGTACCGCCATCGCCCTGATAGACGGTTTTTC (underlined sequence: *Kpn*I). The second step was by ligation of the *amp*^{r} region with the kanamycine resistance gene-deleted pCMVH.gag-Km PCR fragment made using primers 5′-TATACCCGGGCCTGCTATTGTCTTCCCAATCCTCCCCC (underlined sequence: *Xma*I) and 5′-TATAGGTACCGTAAAAAGGCCGCGTTGCTGGCG (underlined sequence: *Kpn*I).

Plasmid pNS.gag was made from plasmid pILH-1which expresses and secretes listeriolysin using the *E. coli* haemolysin secretion elements [16], with the replacement of the listeriosin coding region (*Nco*I–*Nhe*I) with a *Nco*I–*Nhe*I HIV-1 SF2 *gag* gene coding region. The *gag* DNA fragment was made by PCR amplification using pCMVgag template and primers 5′-GTCGACGCCACCATGGGCGCCCGCGC (underlined sequence: *Nco*I) and 5′-GCATCCATGGCTAGCCTGGCTGCTGGGGTCGTTGCCGAAC (underlined sequence: *Nhe*I). Plasmid pNS.*gag* DNA, after removing the *Apa*I restriction enzyme site via cutting with *Apa*I and then filling-in with Klenow enzyme, loses the function of the transport gene *hlyD* and makes a non-secreted Gag protein (our unpublished data and [17]).

2.2. In vitro gene expression

Bacterial cells carrying the HIV gag plasmid were grown in broth to OD_{600} 1–1.2, washed with saline solution twice, and assayed for the level of Gag (p55) protein expression via quantification of p24 peptide, per the Coulter HIV-1 p24 Antigen Assay kit (Coulter Corporation, Miami, FL). Human intestinal Henle monolayers were cultured in Earle's minimal essential medium—2 mM glutamine—10% fetal calf serum, and infected with *Shigella* carrying the HIV gag plasmid as previously described [11]. Mouse bone marrow derived macrophage and dendritic cells were prepared using the protocol by Fortier and Falk [18] and tested for Gag expression as above.

2.3. Immunization and evaluation of bacterial pathogenicity

Bacterial cells grown at late logarithmic phase were washed two times with saline, and resuspended in saline and stored at −80 °C in 10% glycerol (1 OD_{600}/ml). No difference was found between freshly made samples and samples made from the 6-month-old frozen stocks with regard to their immunogenicity in vivo and their ability to invade mammalian cells in vitro.

Female BALB/c and CB6F1 mice were purchased from Charles River Breeding Laboratories (Wilmington, MA) and housed at an AALAC-accredited facility (Chiron, Emeryville, CA). BALB/c mice were used for the Sereny test to evaluate the severity of inflammation caused by *Shigella* strains [19]. CB6F1 mice were used for further assessing the pathogenicity of different mutants in a survival test. Where indicated, mice were sedated by intraperitoneal (i.p.) injection of a mixture of 0.3 mg xylazine hydrochloride (Loyd Labs, Shenandoah, IA), and 1.0 mg ketamine hydrochloride (Fort Dodge Animal Health, Fort Dodge, IA) in 200 μl of saline. Bacteria were administered intranasally (i.n.) (1×10^7 CFU live bacteria in 20 μl saline) and mice were monitored for survival.

2.4. Cellular immune responses

CB6F1 mice were immunized by i.m. injection of pCMV*gag* DNA (50 μl in tibialis anterior muscle) or by i.n. instillation of bacteria (20 μl) then challenged by injection of 1×10^7 PFU of a recombinant vaccinia virus (rVV) encoding the HIV-1 SF2 *gag–pol* genes (rvv*gag–pol*) [14]. Five days after rVV challenge, mouse spleens and lungs were collected, and processed to a single-cell suspension. Gag-specific immune responses were measured by interferon-γ (IFN-γ) production after stimulation with an H-2^d-restricted gag peptide, according to zur Megede et al. [14].

3. Results and discussion

3.1. Selection of an attenuated Shigella carrier

We focused on selection of a *Shigella* carrier from various mutants that were invasive, yet defective in proliferation in or spread between mammalian cells. These mutants were derived from *S. flexneri* SA100 strain, and all have a single gene mutation due to a Tn5 transposon insertion. The genotypes tested included LPS O-antigen synthesis (*rfbF*), membrane

Table 1
Assessment of pathogenicity of bacterial mutants

Strain	Sereny score[a]	Survival[b] (%)
wt	10.2	ND
ipaC	0.5	100
icsA	0.6	20
vpsC	1.8	0
ispA	0.5	40
rfbF	0.1	60
dsbA	ND	20
dapB	ND	0

[a] For the Sereny test, 2.5 μl of a bacterial suspension (6×10^8 CFU) was dripped into one eye; and 2.5 μl of saline, as a control, into the other eye. The mice were observed twice a day for 3 days ($n = 5$ mice per group). The severity of inflammation was scored from 0 to 4 points at each observation: 0 (no inflammation): similar to the negative saline control; 1 (a little inflammation): normal eyelid, wet eye, and blinking frequently; 2 (weak inflammation): wet eye, thick and yellow eyelid, and often closed; 3 (medium inflammation): very thick eyelid, dirty and swollen eye, watery, and usually closed; 4 (strong inflammation): swollen eye, full of secretion, closed all the time, heavy and dirty eyelid. The scores were determined at 4.5, 22, 28.5, 45.5, 52.5, and 72 h, and cumulative values are shown.

[b] For survival after i.n. instillation of 1×10^7 CFU of bacteria values shown indicate endpoint survival ($n = 5$ mice per group).

formation (*vpsC*), cell division (*ispA*), mammalian actin polymerization (*icsA*) [10,11], cell wall synthesis (*dapB*) [12], and protein folding (*dsbA*) [20]. The pathogenicity of the mutant cells was compared after transformation with pCMVH.gag, an HIV-1 SF2 *gag* DNA vaccine. The *rfbF* (pCMVH.gag) cells were found the least reactogenic in the mouse Sereny test [19,21], as no inflammation was observed for any of the five mice (Table 1). In comparison, an acute inflammation of varying degrees was caused by other mutants starting from the first day. Of note, the *ipaC* mutant, though potentially noninfectious in human because of its noninvasive nature [22], caused a detectable inflammation in four of five mice on the first day. Pathogenicity was also assessed with a mouse pulmonary infection model [20,23]. The *rfbF* mutant was again found highly attenuated with 60% of the mice surviving in the bacterial infection (Table 1).

A second criterion for selection was Gag protein expression in infected cells. As shown in Table 2, human intestinal epithelial cells (Henle), and mouse bone marrow derived macrophage and dendritic cells were all found to express Gag within 48 hours after infection with the *rfbF* recombinant carrying pCMVH.gag. The *rfbF* (pCMVH.gag) bacteria alone, without invasion into mammalian cells, expressed a low amount of Gag protein (Table 2), suggesting that pCMVH.gag contains a sequence upstream of the *gag* gene driving protein expression in the prokaryotic cells. In order to determine the contribution of Gag expressed by the *rfbF* (pCMVH.gag) cells in immunogenicity studies, we made a control plasmid (pNS.gag) in which the protein was expressed solely by a prokaryotic expression system.

Table 2
Gag protein expression

Samples	Invasion rate	p24 (pg)
Henle cells	40% (moi, 2:1)	800 (supernatant) 50 (lysate)
Macrophage	80% (moi, 2:1)	31.6 (total)
Dendritic cells	30% (moi, 0.75:1)	358 (total)
rfbF (pCMVH.gag)	NA	5
rfbF (pNS.gag)	NA	10

Shigella rfbF (pCMVH.gag) cells were used to infect three mammalian cells (Henle, macrophage and dendritic cells) at different multiplicity of infection (moi) to obtain high p24 Gag protein expression with limited cell lysis. Invasion rate was defined as the percent of the input mammalian cells infected with at least three bacteria by microscopic detection (see [10]). The input bacteria (3×10^5 CFU) were sedimented onto mammalian cells seeded in a 24-well plate. Expression of p24 Gag protein was assayed by ELISA 48 h after bacterial invasion. For comparison, *Shigella rfbF* cells (carrying pCMVH.gag or pNS.gag) were cultured without the presence of mammalian cells and collected at late log phase of growth. The data represent the averages of three experiments.

3.2. *Immunogenicity of recombinant* Shigella *carrier*

Gag-specific T-cell responses were induced in mice by a single i.n. immunization of live *rfbF* (pCMVH.gag) cells (Table 3). Responses were detected in spleen and lung, as measured by production of INFγ in vitro upon the stimulation of cells with the CD8-restricted H2-k^d Gag peptide p7g. Little or no responses were seen in mice immunized with live *rfbF* cells expressing Gag driven by the prokaryotic promoter (pNS.gag). Therefore, the immune responses elicited by *rfbF* (pCMVH.gag) cells were the result of transfer of the pCMVH.gag plasmid into cells of the host animal. The magnitude of the $CD8^+$ T-cell response was similar to that in mice immunized by i.m. injection of 1–10 ng of naked *gag* DNA. Of note, the estimated amount of the pCMVH.*gag* DNA present in the inoculated *rfbF* (pCMVH.gag) cells was approximately 1–10 ng, indicating similar potency of the two vaccines.

Table 3
$CD8^+$ T-cell responses

Vaccine	Secreted IFN-γ	
	Spleen	Lung
Saline	0	10
rfbF (pNS.gag)	0	12
rfbF (pCMVH.gag)	672	832
DNA 0.1 ng	0	0
DNA 1 ng	0	0
DNA 10 ng	222	2217
DNA 100 ng	1778	2635

Mice were i.n. immunized once with 1×10^6 CFU *Shigella rfbF* cells (carrying pCMVH.gag or pNS.gag) or i.m. with *gag* DNA. Three weeks after immunization, the animals were i.n. challenged with rVV *gag–pol* and 5 days later spleen and lung cells were stimulated in vitro with HIV p7g peptide. The data shown represent the amount of specific IFN-γ production (pg/ml/1×10^6 spleen or lung cells), measured by ELISA, from the supernatants of the cultured cells.

Table 4
Effect of boosting with *Shigella*

Vaccine	Secreted IFN-γ	
	Spleen	Lung
Saline	0	10
gag DNA	852	0
gag DNA × gag DNA	5782	509
gag DNA × *rfbF* (pCMVH.gag)	5527	2870

Mice were i.m. immunized with 0.1 ng DNA followed by no treatment, boosting i.n. with 1×10^6 CFU *Shigella* cells (carrying pCMVH.gag), or boosting with i.m. 0.1 ng DNA. The animals were i.p. challenged with rVV *gag-pol* 3 weeks later and the tissue samples were assayed 5 days after challenge according to the aforementioned methods. The data shown represent average IFN-γ production of two assays (pg/ml/1 × 10^6 spleen or lung cells), as measured by ELISA from culture supernatants.

Immunization with different vaccine modalities in a prime-boost regimen has been tested using various live vector systems (for review see [24]). Here, we tested the boosting potency of Shigella *rfbF* cells (carrying pCMVH.gag) in mice primed with *gag* DNA. We found that the *Shigella* carrier could boost the existing immune response with a potency similar to naked DNA (Table 4). A potential advantage of i.n. administration of the *Shigella* carrier is the induction and boosting of local immune responses in the lung. Indeed, the lung responses were approximately six-fold higher in mice boosted i.n. with *Shigella* versus i.m. with DNA.

3.3. Summary

The use of recombinant bacteria as protein antigen carriers has been described for many years, because of their potential advantages of low cost of commercial production as vaccines and of their high adjuvant activity. Two potential drawbacks are (i) achieving a balance between a high level of antigen expression and the associated toxicity of it in the bacterial hosts and (ii) proteins made in bacteria are often different from proteins made in mammalian cells. This is particularly true for expression of viral antigens. Using recombinant bacteria to deliver plasmid DNA vaccines has the potential to overcome these drawbacks. The results presented here and elsewhere [3,8,9,17,25,26] demonstrate that such vectors can be effective for DNA vaccine delivery for induction of systemic and local immune responses, thus hold promise as recombinant vaccines.

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TAB 3

Zur Megede *et al.*, "Increased expression and immunogenicity of sequence-modified human immunodeficiency virus type 1 gag gene," J Virol. 2000 Mar;74(6):2628-3

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Increased Expression and Immunogenicity of Sequence-Modified Human Immunodeficiency Virus Type 1 *gag* Gene

JAN ZUR MEGEDE, MIN-CHAO CHEN, BARBARA DOE, MARY SCHAEFER, CATHERINE E. GREER, MARK SELBY, GILLIS R. OTTEN, AND SUSAN W. BARNETT*

Chiron Corporation, Emeryville, California 94608

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A major challenge for the next generation of human immunodeficiency virus (HIV) vaccines is the induction of potent, broad, and durable cellular immune responses. The structural protein Gag is highly conserved among the HIV type 1 (HIV-1) gene products and is believed to be an important target for the host cell-mediated immune control of the virus during natural infection. Expression of Gag proteins for vaccines has been hampered by the fact that its expression is dependent on the HIV Rev protein and the Rev-responsive element, the latter located on the *env* transcript. Moreover, the HIV genome employs suboptimal codon usage, which further contributes to the low expression efficiency of viral proteins. In order to achieve high-level Rev-independent expression of the Gag protein, the sequences encoding HIV-1_{SF2} $p55^{Gag}$ were modified extensively. First, the viral codons were changed to conform to the codon usage of highly expressed human genes, and second, the residual inhibitory sequences were removed. The resulting modified *gag* gene showed increases in $p55^{Gag}$ protein expression to levels that ranged from 322- to 966-fold greater than that for the native gene after transient expression of 293 cells. Additional constructs that contained the modified *gag* in combination with modified *protease* coding sequences were made, and these showed high-level Rev-independent expression of $p55^{Gag}$ and its cleavage products. Density gradient analysis and electron microscopy further demonstrated that the modified *gag* and *gagprotease* genes efficiently expressed particles with the density and morphology expected for HIV virus-like particles. Mice immunized with DNA plasmids containing the modified *gag* showed Gag-specific antibody and $CD8^+$ cytotoxic T-lymphocyte (CTL) responses that were inducible at doses of input DNA 100-fold lower than those associated with plasmids containing the native *gag* gene. Most importantly, four of four rhesus monkeys that received two or three immunizations with modified *gag* plasmid DNA demonstrated substantial Gag-specific CTL responses. These results highlight the useful application of modified *gag* expression cassettes for increasing the potency of DNA and other gene delivery vaccine approaches against HIV.

The induction of long-lasting, potent humoral and cellular immune responses will be important for an effective human immunodeficiency virus (HIV) vaccine. Data from HIV-infected patients, and in particular from long-term nonprogressors, have shown that viral structural genes can elicit substantial immune responses. Gag-specific $CD8^+$ cytotoxic T lymphocytes (CTL) have been shown to be important in controlling virus load during acute infection (4, 21) as well as during the asymptomatic stages of the infection (20, 24). Moreover, a strong Gag-specific CTL response appears to correlate inversely with the viral load of HIV-1-infected patients (7). In addition, studies of exposed but uninfected prostitutes indicate that Gag-specific CTL may be involved in protection against the establishment of a persistent HIV type 1 (HIV-1) infection (28). Combined, these studies provide convincing evidence that immune responses directed against HIV Gag proteins may be an important component of an effective HIV vaccine. The usefulness of Gag immunogens for vaccines is further indicated by the fact that the protein is relatively conserved among diverse HIV strains and subtypes, and cross-clade CTL recognition directed against Gag-specific targets has been well documented (2, 3, 11, 23).

Immunization with naked DNA or recombinant virus induces both antibody and CTL responses and has been shown to be an efficient method of eliciting protective immune responses against a broad range of pathogens in animal studies (10). However, the potency of current gene delivery methods such as naked-DNA and viral vectors must be improved to induce adequately robust responses for protection in primates (1). One means to achieve this may be through increasing the expression efficiency of encoded HIV antigens. The poor expression of the HIV structural genes in recombinant vectors is caused by a strong Rev dependency that allows efficient expression only in the presence of the viral Rev protein (25, 30). The translation efficiency and stability of *gag* transcripts are further decreased by the presence of a relatively high AU content and destabilizing AUUUA motifs (inhibitory sequences [INS]). In previous studies, inactivation of these INS enabled the Rev-independent expression of HIV-1 *gag* (29), but these modifications reduced the approximate AT content of the *gag* gene only from 56 to 50%. Elevated percentages of AU in human mRNAs have been shown to result in instability, increased turnover, and low expression levels (15). These findings suggest that further reductions of the AT content of the *gag* gene could result in improved mRNA stability and increased protein expression. In support of this, it has been shown that highly expressed human genes employ codon usage patterns different from those used by HIV genomes. For highly expressed genes, G or C is generally preferred over A or T. Furthermore, changes in the codon usage of HIV-1 *env* to those employed by highly expressed human codons resulted in increased Rev-independent expression (14).

In order to achieve high-level Rev-independent expression

* Corresponding author. Mailing address: Chiron Corporation, Mailstop 4.3, 4560 Horton St., Emeryville, CA 94608. Phone: (510) 923-7565. Fax: (510) 923-2586. E-mail: Susan_Barnett@cc.chiron.com.

of the *gag* gene of HIV-1_{SF2}, the codon usage pattern was first altered to conform to that used by highly expressed human genes (14). Further modifications were then made to remove possible residual INS motifs previously identified in the *gag* coding region (29). This resulted in lowering the AT content of the *gag* coding sequences from 56 to 32%, a level more consistent with increased mRNA stability and translation efficiency. The sequence-modified HIV-1_{SF2} *gag* gene was inserted into a high-level plasmid expression vector for in vitro transfections and DNA immunization studies with rodents and nonhuman primates. Results presented here indicate that sequence-modified *gag* plasmids expressed protein at dramatically higher levels and showed increased immunogenicity compared to the native *gag* sequence in DNA immunization experiments performed with mice and rhesus macaques. Additionally, the inclusion of modified protease-coding sequences in the modified *gag* resulted in high-level Rev-independent expression, processing of the Gagprotease polyprotein, and the production of virus-like particles (VLP) with the morphologies of both immature and mature HIV-1 virions.

MATERIALS AND METHODS

***gag* and *gagprotease* plasmids.** The native sequences coding for the 502 amino acids (aa) of HIV-1_{SF2} $p55^{Gag}$ (GenBank accession no. K02007) were modified to change the codon usage to that utilized by highly expressed human genes as described recently for HIV-1_{MN} gp120 (14). In addition, regions with INS were further inactivated without altering the reading frame for the $p55^{Gag}$ nucleic acid sequence. The resulting modified HIV-1_{SF2} *gag* encoded a $p55^{Gag}$ protein with three amino acid changes (Asn377Thr, Ile403Thr, and Lys405Arg); the resulting amino acid sequence conformed to the sequences for other HIV-1 subtype B Gag proteins in the HIV sequence database (Los Alamos National Laboratory; http://hiv-web.lanl.gov/cgi-bin/hivDB3/public/wdb/ssampublic) (GenBank accession no. AF201927). To further enhance the translation efficiency of the modified *gag*, an optimal consensus sequence for the initiation of translation (GCCACC AUGG) was employed (22). The resulting 1,527-bp gene cassette included the *Sal*I and *Eco*RI cloning sites and was constructed synthetically by the Midland Certified Reagent Company (Midland, Tex.). This modified *gag* sequence was cloned into the *Sal*I and *Eco*RI restriction sites of the eukaryotic expression vector pCMVKm2 that employs the cytomegalovirus (CMV) immediate-early enhancer/promoter and bGH terminator (Chiron Corporation, Emeryville, Calif.) (6), resulting in the plasmid pCMVKm2.GagMod.SF2. For the comparison of expression efficiencies between the modified and the native HIV-1_{SF2} *gag* expression cassettes, three different vectors containing the native $p55^{Gag}$ coding sequence were used, pCMV6ap55GagPRE, pCMVKm2p55GagPRE, and pCMVLinkPREp55Gag (Chiron). The pCMVLink plasmid differs from pCMVKm2 only in its multiple cloning site. All of these use the CMV immediate-early enhancer/promoter and include the hepatitis B virus posttranscriptional regulatory element (PRE) (9, 16–18) to partially overcome the Rev dependency of *gag*. This was demonstrated by transfection experiments using the native HIV-1_{SF2} *gag* gene with and without PRE. The expression of $p55^{Gag}$ was clearly improved using PRE over that using the *gag* gene only (S. W. Barnett, unpublished data).

For the construction of the *gagprotease* expression cassettes, modifications were made in the same manner as that described for *gag* up to the −1 frameshift region of the *pol* gene. The sequence from there to the *gag* gene's stop codon was unaltered. The sequences from the *gag* stop codon to the codons for first 26 aa of the reverse transcriptase were codons either optimized with subsequent INS inactivation as described above (GP1; GenBank accession no. AF202464) or modified by INS inactivation alone (GP2; GenBank accession no. AF202465). Both versions of the *gagprotease* cassette were cloned into the pCMVKm2 vector as described above for the modified *gag* to yield the plasmids pCMVKm2.GagProtMod.SF2 (GP1) and pCMVKm2.GagProtMod.SF2 (GP2).

In vitro expression assays. Plasmid DNA was purified using endotoxin-free columns (Qiagen, Valencia, Calif.). African green monkey kidney (COS-7; European Culture Collections Organization no. 87021302), human kidney (293; American Type Tissue Collection [ATCC; Atlanta, Ga.] no. 45504), and human rhabdomyosarcoma (RD; ATCC no. CCL-136) cells were plated 1 day prior to transfection at a density of 5×10^5 cells per 35-mm-diameter well (Corning). For the transfections, 2 μg of each plasmid DNA was mixed with the Mirus TransIT-LT1 polyamine transfection reagent (PanVera, Madison, Wis.). The green fluorescent protein (GFP) reporter gene vector pEGFP (Clontech, Palo Alto, Calif.) was used as a transfection efficiency control in co- and parallel transfections. The cells were incubated with 2 ml of medium per well (for 293 cells, Iscove's modified Dulbecco's medium, 10% fetal calf serum [FCS]; for COS-7 and RD cells, Dulbecco's modified Eagle medium, 10% FCS; Gibco, Rockville, Md.). To estimate the transfection efficiency, GFP-expressing cells were analyzed quantitatively by flow cytometry (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) and directly counted under a fluorescence microscope. Supernatants were harvested 24, 48, and 60 h posttransfection and filtered through 0.45-μm-pore-size syringe filters (Pall Corp., Ann Arbor, Mich.). Cells were harvested 60 h posttransfection, washed twice in phosphate-buffered saline (PBS) and then lysed on ice in 40 μl of buffer containing 1% NP-40 (Sigma, St. Louis, Mo.) and 0.1 M Tris-HCl, pH 7.5. Cell lysates were subsequently clarified by centrifugation in an Eppendorf microcentrifuge at 4°C for 10 min to remove cellular debris. The quantitation of Gag p24 protein in cell supernatants and lysates was performed using the p24 antigen capture enzyme-linked immunosorbent assay (ELISA) (Coulter Corporation, Miami, Fla.). For immunoblot analysis, samples were electrophoresed through sodium dodecyl sulfate (SDS)–8 to 16% polyacrylamide gels (Novex, San Diego, Calif.) and then transferred onto Immobilon P membranes (Millipore, Bedford, Mass.). A prestained broad-range molecular weight marker (Bio-Rad, Hercules, Calif.) and the HIV-1 p24 protein (Chiron) were used as size standards. Membranes were then incubated with HIV-1 patient serum or mouse anti-p24 monoclonal antibody (MAb) 76C.5EG (Chiron) (31). Reactive bands were visualized using Sigma Fast 3,3′-diaminobenzidine substrate as described by the manufacturer.

Sucrose density gradients. Supernatants from transfected 293 and COS-7 cells were collected at 24 and 48 h posttransfection, filtered through a 0.2-μm-pore-size filter, and concentrated by ultracentrifugation through a 20% (wt/wt) sucrose cushion for 2 h at 140,000 × *g* (24,000 rpm) using a Beckman SW28 rotor. The pellets were then suspended in PBS, loaded on a 20 to 60% sucrose gradient, and centrifuged at 285,000 × *g* (40,000 rpm) for 2 h in a Beckman SW41ti rotor. Each gradient was fractionated into 1-ml aliquots, and 10-μl aliquots of each fraction were electrophoresed on an SDS–8 to 16% polyacrylamide gel electrophoresis gel (Novex). In addition, 2.5 μl of the concentrated gradient preload material was also analyzed. The proteins were then transferred to Immobilon P membranes (Millipore) and probed with mouse anti-p24 MAb 76C.5EG at a dilution of 1:2,000.

Electron microscopy. COS-7 or 293 cells (4×10^6) were transfected in 100-mm-diameter dishes (Corning), and cells were harvested at 24 or 48 h posttransfection. Cells transfected with vector DNA alone served as negative controls. After two washes with PBS the cells were fixed in 2% glutaraldehyde (Sigma), incubated for 20 min at room temperature, gently scraped from the plate, and transferred into a 15-ml polypropylene tube. The fixed cells were then stained with uranium acetate and lead citrate. Electron microscopy was carried out using a transmission electron microscope (Zeiss; 10c) at ×50,000 and ×100,000 magnifications.

Animal studies. Female BALB/c and CB6F1 mice, 6 to 8 weeks old, were used for immunogenicity studies. For the first experiment (Fig. 5), four groups of BALB/c mice ($n = 4$) were immunized with either modified *gag* plasmid DNA (pCMVKm2.GagMod.SF2) or the native *gag* plasmid DNA (pCMV Link.Gag.SF2.PRE). The plasmid DNA doses for the different groups were 20, 2, 0.2, and 0.02 μg in 100 μl of sterile endotoxin-free saline (Sigma). pCMVKm2 vector DNA was used to maintain the total concentration of DNA in each dose at 20 μg/100 μl to control for effects due to the lower concentration of plasmid DNA (2-, 0.2-, and 0.02-μg doses). For experiments 2 and 3 shown in Fig. 6 and 7, the mouse strain employed was CB6F1. For experiment 3, plasmid DNA doses were further diluted to include doses as low as 0.0002 μg.

For the DNA immunization study with rhesus monkeys (*Macaca mulatta*), four animals were immunized bilaterally in the quadriceps muscles with 1-mg doses of pCMVKm2.GagMod.SF2 plasmid DNA in saline at weeks 0, 4, and 8 and bled at weeks 0, 4, 6, 8, and 10. Animals were maintained at the Southwest Foundation for Biomedical Research (San Antonio, Tex.).

Measurements of antibody responses. Ninety-six-well plates (Corning) were coated with 100 μl of recombinant HIV-1_{SF2} p24 antigen at a concentration of 2 μg per ml in 50 mM borate buffer, pH 9. Sera were diluted 1:25, followed by threefold serial dilutions in dilution buffer containing 1% casein as the blocking reagent. Pooled anti-p24 antibody-positive mouse sera served as both a positive control and an assay standard. The sera were incubated for 50 min at 37°C, washed, and incubated with a 1:22,000 dilution of goat anti-mouse immunoglobulin G (IgG)-IgM peroxidase conjugate (Pierce, Rockford, Ill.) for an additional 50 min at 37°C. After the plates were washed, the tetramethylbenzone substrate (Pierce) was added to each well, and the reaction was stopped after 30 min by the addition of 2 N H_2SO_4. The plates were read on an ELISA reader (312e; Bio-Tek, Winooski, Vt.) at 450 nm with a reference wavelength of 620 nm. The calculated titers are the reciprocal of the dilution of serum at a cutoff optical density of 0.4.

Recombinant vaccinia virus challenge of immunized mice. The recombinant vaccinia virus containing the HIV-1_{SF2} *gag* and *pol* genes (rVV*gag-pol*) has been described previously (8). Nine (experiment 2, Fig. 6) and 5 (experiment 3, Fig. 7) weeks following *gag* DNA immunization, mice were challenged with an intraperitoneal injection of 10^7 PFU of rVV*gag-pol*. Five days later spleens were harvested and tested directly for cytolytic activity against Gag peptide-pulsed, ^{51}Cr-labeled tumor target cells or were stimulated with Gag peptide and then stained for intracellular gamma interferon (IFN-γ), as described below. This rVV*gag-pol* challenge model provides a quantitative measure of $CD8^+$ T-cell function (G. Otten, unpublished data).

CTL assays. Spleen cells were tested for cytolytic activity in a 4-h ^{51}Cr release assay using ^{51}Cr-labeled SVBALB (H-2^d) or RMA (H-2^b) tumor target cells (5,000 targets per well) that had been pulsed for 1 h with a 1-μg/ml concentration

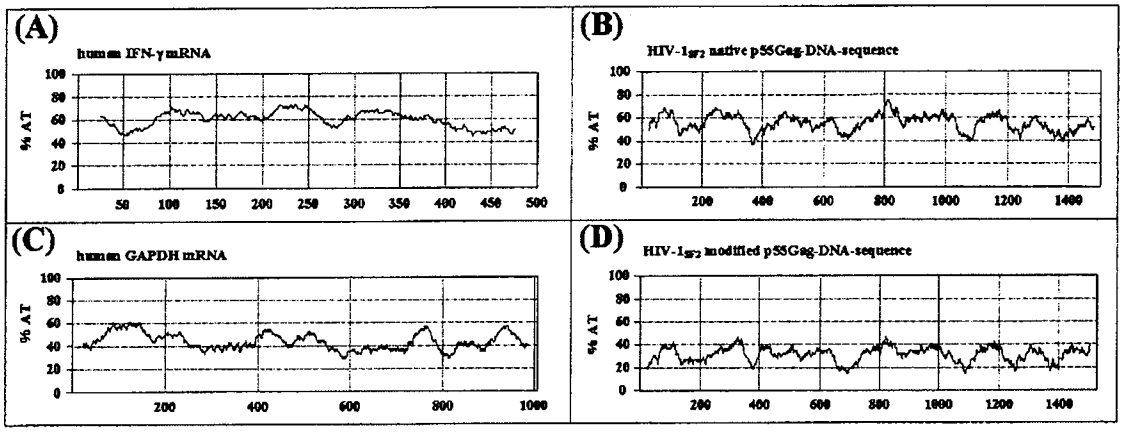

FIG. 1. Comparison of the percentages of A and T nucleotides in genes encoding relatively unstable versus stable mRNA molecules. The human IFN-γ gene and the native HIV-1_{SF2} *gag* DNA sequences both encode relatively unstable transcripts (A and B) and have an average AT content of 55 to 60%. In contrast, the stable human GAPDH gene and the modified HIV-1_{SF2} *gag* coding regions have reduced AT contents of 40 and 30%, respectively (C and D). The calculation of the AT content was done using MacVector software (Oxford Molecular Ltd.); the window size was set at 50.

of the H-2K^d-binding HIV-1 Gag peptide p7g (8) or the control HIV-1 Gag peptide pgag^b (12, 26). After 4 h of incubation, 50 μl of culture supernatants was transferred to Lumaplates (Packard, Meriden, Conn.), dried, and counted in a Microbeta scintillation counter (Wallac, Gaithersburg, Md.). Percent specific ^{51}Cr release was determined from the formula percent specific ^{51}Cr release = (mean experimental release − mean spontaneous release)/(maximum release − spontaneous release) × 100%, where spontaneous release = mean counts per minute released from target cells in the absence of spleen cells and maximum release = mean counts per minute released from target cells in the presence of 0.1% Triton X-100.

Measurement of Gag-specific IFN-γ-producing CD8$^+$ lymphocytes. Spleens were taken 5 days post-rVV*gag-pol* challenge. Erythrocyte-depleted single cell suspensions were prepared by treatment with Tris-buffered NH_4Cl (Sigma). Nucleated spleen cells (1×10^6 to 2×10^6) were cultured in duplicate at 37°C in the presence or absence of 10 μg of p7g peptide/ml. Monensin (Pharmingen, San Diego, Calif.) was added to block cytokine secretion. After 3 to 5 h cells were washed, incubated with anti-CD16/32 (Pharmingen) to block Fcγ receptors, and fixed in 2% (wt/vol) paraformaldehyde and stored overnight at 4°C. The following day cells were treated with 0.5% (wt/vol) saponin (Sigma) and then incubated with a phycoerythrin (Pharmingen)-conjugated mouse IFN-γ MAb in the presence of 0.1% (wt/vol) saponin. Cells were then washed free of saponin, stained with fluorescein isothiocyanate-conjugated CD8 MAb (Pharmingen), washed, and then analyzed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems). Samples were cultured and stained in duplicate.

Peptide pools. A set of 51 Gag peptides 20 residues long, overlapping by 10 aa and spanning residues 1 to 496 of HIV-1_{SF2} p55^{Gag}, was synthesized (Chiron Mimotopes, Clayton, Australia). Eight pools were made by mixing 5 to 7 overlapping peptides. Gag amino acid sequences spanned by the pools were as follows: aa 1 to 80, pool 1; aa 71 to 144, pool 2; aa 135 to 203, pool 3; aa 194 to 263, pool 4; aa 254 to 323, pool 5; aa 314 to 365, pool 6; aa 351 to 430, pool 7; aa 421 to 496, pool 8. A pool of six 20-aa overlapping peptides representing HIV-1_{SF2} Env served as a negative-control pool.

Purification of rhesus macaque PBMC and derivation of B-LCL. Rhesus macaque peripheral blood mononuclear cells (PBMC) were separated from heparinized whole blood on Percoll gradients (5) and cultured at 3×10^6 to 3.5×10^6 per well in 1.5 ml in 24-well plates for 8 days in AIM-V–RPMI 1640 (50:50) culture medium (Gibco) supplemented with 10% FCS. Gag-specific cells were stimulated by the addition of either a Gag peptide pool (13.3 μg of total peptide/ml) or autologous PBMC that had been infected with rVV*gag-pol* and cultured in 24-well plates. Recombinant human interleukin-7 (IL-7; 15 ng/ml; R&D Systems, Minneapolis, Minn.) was added at the initiation of culture. Human recombinant IL-2 (20 IU/ml; Proleukin; Chiron) was added on days 1, 3, and 6. For the derivation of stable rhesus B-lymphoblastoid cell lines (B-LCL), PBMC were exposed to herpesvirus papio-containing culture supernatant from the S594 cell line (13, 27) in the presence of 1 μg of cyclosporine (Sigma)/ml.

Rhesus macaque CTL assay. Autologous B-LCL were labeled overnight with $Na_2{}^{51}CrO_4$ (NEN, Boston, Mass.; 25 μCi per 10^6 B-LCL) and washed. Individual aliquots were then incubated for 1 h with 100 μg of Gag or Env peptide pool/ml. Peptide-pulsed, ^{51}Cr-labeled B-LCL were added (2,500 per round-bottom well) to duplicate wells containing threefold serial dilutions of cultured PBMC. Unlabeled B cells (10^5) were added to each well to inhibit nonspecific cytolysis. After 4 h, 50 μl of culture supernatant was harvested, added to Lumaplates (Packard), and counted with a Microbeta 1450 liquid scintillation counter (Wallac). ^{51}Cr released from lysed targets was normalized by the formula percent specific ^{51}Cr release = 100% × (mean experimental release − mean spontaneous release)/(maximum release − spontaneous release), where spontaneous release = mean counts per minute released from target cells in the absence of spleen cells and maximum release = mean counts per minute released from target cells in the presence of 0.1% Triton X-100. Data are plotted as percent specific ^{51}Cr release versus the culture fraction, where the culture fraction represents the fraction of the culture well (1.5 ml) added to the CTL assay microtiter plate, e.g., a culture fraction of 0.067 equals 1/15 or 0.1 ml of the initial PBMC culture. Serial threefold dilutions of the cultured PBMC were made. In separate experiments, where we have counted the cells recovered from cultures, we have determined the maximal effector cell/target cell ratios to be about 40:1 to 100:1.

RESULTS

Increased in vitro expression efficiency of sequence-modified HIV-1_{SF2} *gag* gene. The coding sequences for the HIV-1_{SF2} *gag* gene were modified to conform to the codon usage pattern of highly expressed human genes and to eliminate residual INS motifs as described in Materials and Methods. These modifications resulted in *gag* coding sequences with a clear reduction in overall AT content compared to that of the native *gag* (Fig. 1). In fact, the percentage of A and T nucleotides was reduced from 56 to 32%, a level more consistent with increased mRNA stability and translation efficiency (14, 15). The AT content of the modified *gag* more closely resembled that of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, which encodes a relatively stable mRNA compared with the relatively unstable AU-rich human IFN-γ mRNA (Fig. 1).

The in vitro expression efficiency of the modified HIV-1_{SF2} *gag* (pCMVKm2.GagMod.SF2) was compared to that of the native SF2 *gag* in a construct (pCMVLink.Gag.SF2.PRE) that also contained the hepatitis B virus PRE. The pCMVLink.Gag.SF2.PRE plasmid previously had been found to express Gag at substantially higher levels than a similar plasmid containing the HIV-1_{SF2}-derived *gag* gene without the PRE (S. W. Barnett, unpublished data). The expression levels for these plasmids were determined in several independent experiments after transfection of three different cell lines, RD, 293, and COS-7 (Table 1). Cell supernatants and lysates were tested at 48 and 60 h posttransfection. Gag expression levels were clearly much higher for the modified *gag* plasmid at all time points and in all three cell lines tested. The increased expression was most dramatic in the supernatants of the transfected human 293 cell line, where expression from the modified *gag*

TABLE 1. Increased in vitro expression from modified versus native *gag* plasmids in supernatants and lysates from transiently transfected cells

Experiment	Type of plasmid[a]	Material assayed[b]	Cell line	Hours post-transfection	Total ng p24 (fold increase)
1	Nat	Sup	293	48	3.4
	Mod	Sup	293	48	1,260 (371)
	Nat	Sup	293	60	3.2
	Mod	Sup	293	60	2,222 (694)
2	Nat	Sup	293	60	1.8
	Mod	Sup	293	60	1,740 (966)
3	Nat	Sup	293	60	1.8
	Mod	Sup	293	60	580 (322)
4	Nat	Lys	293	60	1.5
	Mod	Lys	293	60	85 (57)
1	Nat	Sup	RD	48	5.6
	Mod	Sup	RD	48	66 (12)
	Nat	Sup	RD	60	7.8
	Mod	Sup	RD	60	70.2 (9)
2	Nat	Lys	RD	60	1.9
	Mod	Lys	RD	60	7.8 (4)
1	Nat	Sup	COS-7	48	0.4
	Mod	Sup	COS-7	48	33.4 (84)
2	Nat	Sup	COS-7	48	0.4
	Mod	Sup	COS-7	48	10 (25)
	Nat	Lys	COS-7	48	3
	Mod	Lys	COS-7	48	14 (5)

[a] Nat, native (pCMVLink.Gag.SF2.PRE); Mod, modified (pCMVKm2.Gag-Mod.SF2).
[b] Sup, supernatant; Lys, lysate.

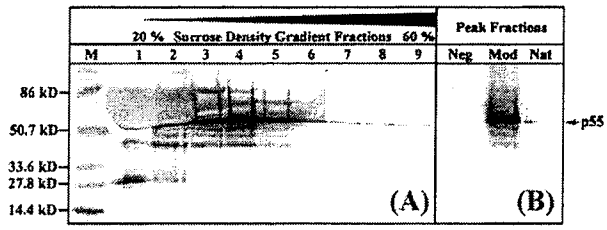

FIG. 2. Increased expression in vitro of HIV-1_{SF2} $p55^{Gag}$ particles in cells transfected with the modified *gag* gene. 293 cells were transfected with plasmids containing either the modified or native HIV-1_{SF2} *gag* genes. Supernatants from transfected cell cultures were collected at 60 h posttransfection and centrifuged through 20 to 60% sucrose density gradients. Gradient fractions were collected, run on an SDS–8 to 16% polyacrylamide gel, and analyzed by Western blotting as described in Materials and Methods. (A) Immunoblot of fractions 1 to 9 from the sucrose density gradient from transfection supernatants of the modified *gag* plasmid. (B) Immunoblot comparing peak fractions collected in the density range expected for HIV-1 VLP after transfection with modified (Mod) or native (Nat) HIV-1_{SF2} *gag* plasmids. Vector alone (Neg) was used as a negative transfection control, and the prestained broad-range molecular weight marker (M) was used as the size standard.

was 322- to 966-fold greater than that of the native HIV-1_{SF2} *gag* plasmid tested. The improvement in Gag expression levels in 293 cell lysates was also apparent, but less so than in the supernatants, which could be indicative of more-efficient budding of $p55^{Gag}$ particles in cells where expression levels are elevated. To exclude possible effects on the transfection efficiency depending on the plasmid used, flow cytometry and direct fluorescence microscope analysis were done in parallel transfections or by cotransfection using GFP plasmid DNA. On average, 70% of the cells were transfected using either method with no differences in transfection efficiency between the native and modified *gag* plasmids noted (data not shown).

The modified HIV-1_{SF2} *gag* gene encodes $p55^{Gag}$ VLP of the expected density and morphology. Supernatants and cell lysates from transfected 293 cells were subjected to immunoblot and density gradient sedimentation analysis. The results confirmed the previous data from the p24 capture assay with respect to the relative level of p55 expression from the modified *gag* plasmid. The expected $p55^{Gag}$ band was detected using human HIV-1 patient serum (Fig. 2) or an anti-p24 MAb for the immunostaining (data not shown). Supernatants from 293 cells transfected with the native and modified *gag* genes were subjected to rate zonal sedimentation to isolate $p55^{Gag}$ particles of the reported density (32). Gradient fractions were analyzed by p24 capture ELISA (data not shown) and Western blotting (Fig. 2A) to determine the peak fraction of each sample. Western blot analysis showed that the $p55^{Gag}$ band for the modified Gag expression cassette was stronger than that for the best native *gag* plasmid (Fig. 2B).

To confirm that VLP were being expressed, COS-7 cells transfected with pCMVKm2.GagMod.SF2 were harvested at 24 h posttransfection and electron microscopy was performed. As shown in Fig. 3A, budding and free immature particles could be observed. These data confirm that the sequence modifications for the *gag* gene did not adversely affect the $p55^{Gag}$ particle assembly or VLP morphology.

Construction and characterization of sequence-modified *gagprotease* gene cassettes. As a first step in the design of modified HIV immunogens with increased representation of Pol-specific epitopes, two different modified *gagprotease* gene constructs were evaluated for expression and VLP formation. The protease coding sequences in these constructs were (i) codon optimized, with subsequent INS inactivation as described above for *gag* (GP1), or (ii) modified by INS inactivation alone (GP2). Like the modified *gag* plasmid, in the absence of Rev both versions of the modified *gagprotease* exhibited high-level expression of Gag proteins in supernatants and cell lysates of transiently transfected COS-7 and 293 cell lines (Table 2). In fact, the expression levels measured in lysates of 293 cells transfected with the *gagprotease* plasmids were higher than those seen with the modified *gag* alone. This result could be partially or wholly attributed to more-efficient recognition of processed Gag (mostly p24) than of unprocessed $p55^{Gag}$ by the Coulter p24 antigen capture assay, as has been previously described (29). This apparent increase in p24 expression in cell lysates was not observed in COS-7 cells, possibly due to lower overall expression of $p55^{Gag}$ in this cell line.

Sucrose density gradient analyses of supernatants from 293 and COS-7 cells transiently transfected with either *gagprotease* or *gag* constructs were performed, and the peak fractions were subsequently analyzed by Western blotting. The efficiency of VLP formation varied between the cell lines tested and was found to be lower for *gagprotease* than for the modified *gag* plasmid (Fig. 4). The levels of VLP formation from the two *gagprotease* constructs in 293 cells were similar (Fig. 4A; GP1 and GP2), but the analysis of the codon-optimized and INS-inactivated *gagprotease* plasmid, GP1, in COS-7 cells suggested the production of relatively small amounts of VLP (Fig. 4B). Polyproteins expressed from both of the modified versions of *gagprotease* were correctly processed by the encoded viral protease. Bands corresponding to unprocessed $p55^{Gag}$ and completely processed p24 were detectable using a MAb specific for p24 (data not shown) or HIV-1^+ patient serum (Fig. 4A and B) (p17 levels were too low to be detected with the HIV^+ sera used).

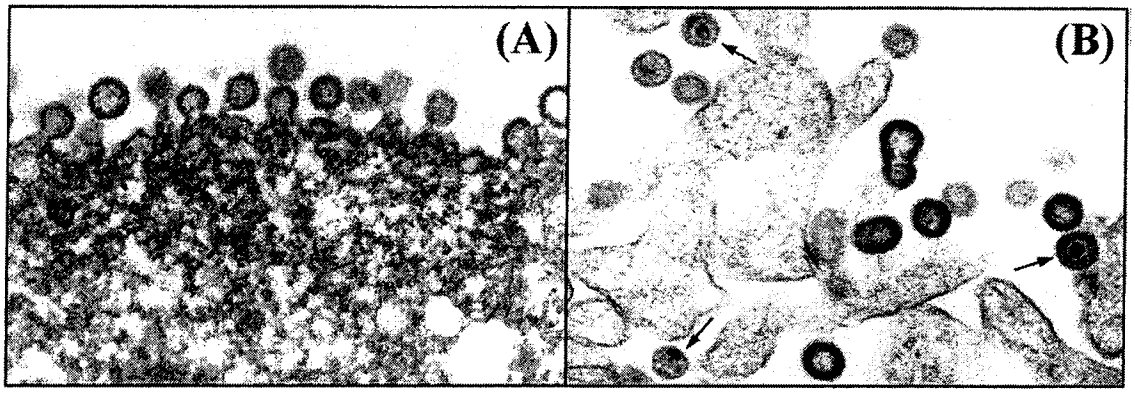

FIG. 3. Modified *gag* and *gagprotease* form VLP in transiently transfected COS-7 cells. Shown are electron micrographs of immature $p55^{Gag}$ VLP in COS-7 cells transfected with the modified HIV-1_{SF2} *gag* (A) and mature (arrows) and immature VLP obtained using the modified HIV-1_{SF2} *gagprotease* (GP2) (B). Transfected cells were fixed at 24 (*gag*) or 48 h (*gagprotease*) posttransfection and subsequently analyzed by electron microscopy as described in Materials and Methods (magnification, ×100,000). Cells transfected with vector alone (pCMVKm2) served as the negative control (data not shown).

Electron microscopic analysis of COS-7 cells transfected with the two different sequence-modified *gagprotease* constructs confirmed the results of the sucrose gradient analysis. COS-7 cells transfected with the codon-optimized and INS-inactivated version (GP1) showed very little VLP formation (data not shown) compared to those transfected with the non-optimized INS-inactivated *gagprotease* (GP2; Fig. 3B). A possible explanation for this observation is that codon optimization of the *protease* coding sequences may have resulted in its overexpression relative to Gag and the prevention of the efficient budding of particles (19). The GP2 version of *gagprotease*, in which the INS of the protease-coding region was inactivated without codon optimization, reduced protease overexpression, and thus VLP of the mature and immature phenotypes could be detected.

Increased immunogenicity of the modified *gag* DNA in vivo. To evaluate and compare the immunogenicities of the modified and the native *gag* plasmids, mice were immunized intramuscularly with plasmid DNA doses titrated from 20 to 0.02 μg per mouse. Serum was collected at 4 weeks postimmunization and tested in a $p24^{Gag}$-specific antibody ELISA. Antibody responses to Gag could be detected in mice immunized with as little as 0.2 μg of the modified *gag* expression cassette, whereas the native *gag* cassette was able to induce an antibody response only at the 20-μg DNA dose (Fig. 5A). This represented the induction of an antibody response using the modified *gag* at a single DNA dose 100 fold lower than that necessary for the native *gag*. In parallel groups of animals, a second dose of DNA was given at 4 weeks to determine if antibody responses to the modified *gag* had reached maximal values at the 20-μg dose and if the lowest DNA dose of 0.02 μg could induce an antibody response after a second immunization. As shown in Fig. 5B, the Gag-specific antibody titers increased after the second immunizations for all DNA doses except for the 0.02-μg DNA dose group, which remained negative.

Measurements of the cellular immune responses following DNA immunization with the modified *gag* demonstrated a similar pattern. Gag-specific CTL responses were inducible at DNA amounts at least 10-fold lower than those necessary with

TABLE 2. In vitro expression from modified *gag* and *gagprotease* plasmids in supernatants and lysates from transiently transfected cells

Plasmid[a]	Material assayed[b]	Cell line	Hours posttransfection	Total ng p24[c]
Gag	Sup	293	60	760
GagProt (1)	Sup	293	60	380
GagProt (2)	Sup	293	60	320
Gag	Lys	293	60	78
GagProt (1)	Lys	293	60	1,250
GagProt (2)	Lys	293	60	400
Gag	Sup	COS-7	72	40
GagProt (1)	Sup	COS-7	72	150
GagProt (2)	Sup	COS-7	72	290
Gag	Lys	COS-7	72	60
GagProt (1)	Lys	COS-7	72	63
GagProt (2)	Lys	COS-7	72	58

[a] Gag, pCMVKm2.GagMod.SF2; GagProt (1), pCMVKm2.GagProtMod.SF2 (GP1) (*gagprotease* with codon optimization and inactivation of INS in *protease*); GagProt (2), pCMVKm2.GagProtMod.SF2 (GP2) (*gagprotease* with only inactivation of INS in *protease*).

[b] Sup, supernatant; Lys, lysate.

[c] Representative results from three independent experiments for each cell line tested.

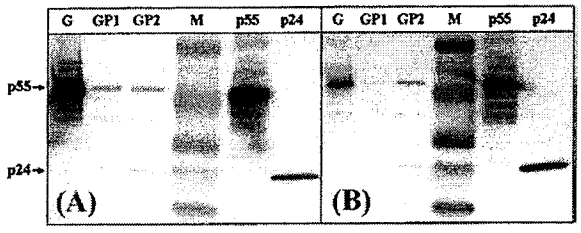

FIG. 4. Expression and processing of $p55^{Gag}$ polyproteins in VLP using modified HIV-1 *gagprotease*. Supernatants from transfected cell cultures were collected at 60 h posttransfection and centrifuged through 20 to 60% sucrose density gradients. Gradient fractions were collected, and peak fractions were run on an SDS–8 to 16% polyacrylamide gel and analyzed by Western blotting using HIV-1 patient serum as described in Materials and Methods. (A) Peak fractions from 293 cells. Results for the modified *gag* (G) are compared to those for codon-optimized, INS-inactivated *gagprotease* (GP1) and for INS-inactivated-only *gagprotease* (GP2). (B) Immunoblot comparing peak fractions from transfected COS-7 cells using the same plasmids as those described for panel A. Purified HIV-1_{SF2} p24 (Chiron) and baculovirus-derived $p55^{Gag}$ proteins were used as additional controls. Prestained broad-range markers (Bio-Rad) were used as size standards (M).

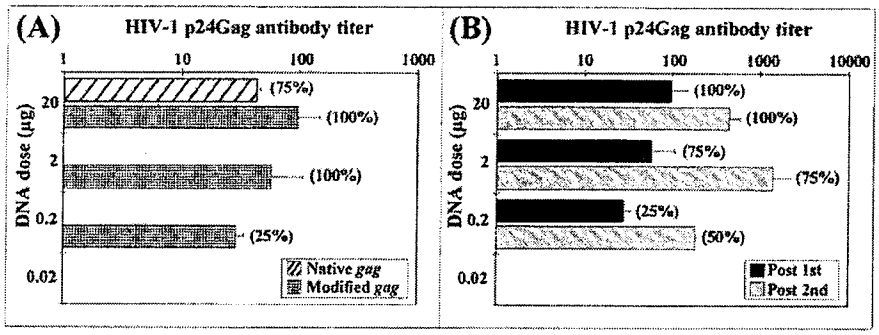

FIG. 5. Increased immunogenicity of the modified HIV-1_{SF2} *gag* plasmid compared to that of the native *gag* plasmid. Groups of mice were immunized bilaterally in the tibialis anterior muscles with titrated amounts of DNA in 10-fold dilutions from 20 μg down to 0.002 μg. Sera were collected at 0 and 4 weeks and tested for HIV-1 p24-specific antibody titers by ELISA as described in Materials and Methods. (A) Comparison of humoral immune responses at different DNA doses using the native and modified *gag* plasmid DNA. Values represent the geometric mean antibody titers and the standard deviations of the midpoint antibody titers for each group. The values in parentheses indicate the percentages of responders (percent seroconversion) in each group. (B) Antibody responses were boosted following a second immunization with the modified *gag* plasmid DNA. Four weeks after the first immunization, additional groups of mice received a second immunization with the same amount of titrated plasmid DNA. Sera collected at weeks 0, 4, and 6 were analyzed by p24 antibody ELISA.

the native *gag* expression cassette (Fig. 6). Gag-specific CTL were detectable after a single immunization with a dose of the modified *gag* plasmid DNA as low as 0.02 μg, whereas a dose of 0.2 μg of the native *gag* plasmid was required for the induction of detectable CTL. In a subsequent study, the modified *gag* plasmid DNA was further diluted (down to 0.2 ng) and used to immunize additional groups of mice. As shown in Fig. 7, Gag-specific IFN-γ-positive $CD8^+$ T cells were scored in mice receiving as little as 2 ng of the modified *gag* DNA.

Induction of CTL responses in rhesus macaques immunized with the modified *gag* plasmid. Based on the increased potency observed in mouse immunizations with the modified *gag* plasmid DNA, studies with nonhuman primates were initiated. Four rhesus macaques were given three intramuscular immunizations with 1-mg doses of *gag* plasmid at 4-week intervals. PBMC were harvested prior to immunization and at 2 weeks after the second and third immunizations. PBMC were cultured with Gag peptide pools or with rVV*gag-pol*-infected autologous PBMC to stimulate the expansion and differentiation of CTL and tested against Gag peptide pool-pulsed, ^{51}Cr-labeled, autologous B-LCL targets in 4-h ^{51}Cr release assays. No Gag-specific cytolysis in PBMC was observed prior to immunization (not shown). However, after *gag* DNA immunization, all four macaques showed cytolytic activity against autologous B-LCL pulsed with at least one Gag peptide pool. In addition, two of the four macaques reacted with two or three Gag peptide pools (Fig. 8). Percent specific lysis of Gag-pulsed target cells varied among animals and among pools and reached as high as 80% at the highest effector cell/target cell ratio (Fig. 8C). A Gag-specific antibody response (antibody titer, 164) was detected in one of the four animals 2 weeks after the second immunization. This animal also had an anamnestic immune response 2 weeks after the third immunization, with a fivefold increase of the antibody titer (890). A second animal

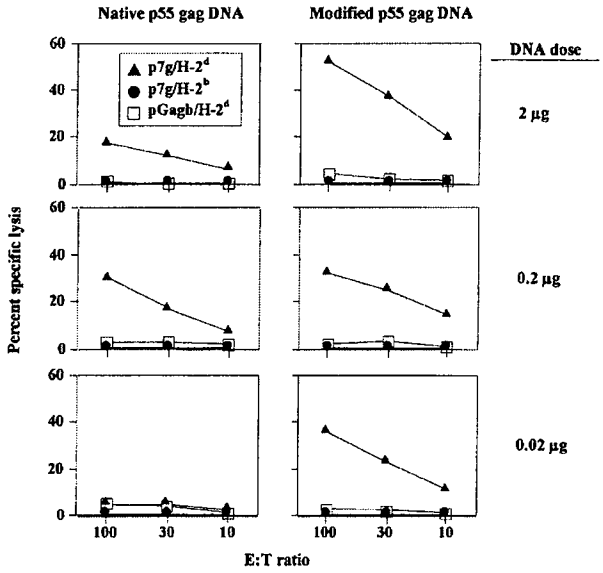

FIG. 6. CTL responses in CB6F1 mice after a single immunization with titrated plasmid DNA. Nine weeks after immunization mice were challenged with an intraperitoneal dose of 10^7 PFU of rVV*gag-pol*. Five days later effector (E) spleen cells were tested for cytolytic activity in a 4-h ^{51}Cr release assay using ^{51}Cr-labeled SVBALB tumor target (T) cells (5,000 targets per well) that had been pulsed for 1 h with a 1-μg/ml concentration of the H-2K^d-binding HIV-1 Gag peptide p7g (▲). Target cells pulsed with the negative-control HIV-1 Gag peptide pgag^b (□) and major histocompatibility complex-mismatched (H-2^b), p7g peptide-pulsed RMA target cells (●) were employed as negative controls.

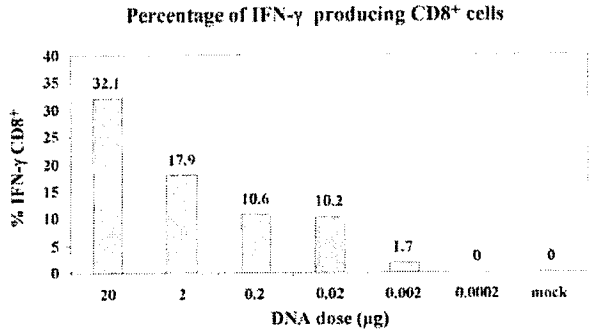

FIG. 7. Quantification of Gag-specific, IFN-γ-producing $CD8^+$ T lymphocytes in mice after a single immunization of titrated modified *gag* plasmid DNA followed by rVV*gag-pol* challenge. Splenic IFN-γ-positive $CD8^+$ T lymphocytes specific for the p7g Gag peptide were enumerated by flow cytomerty as described in Materials and Methods. mock, results using spleen cells from naive mice.

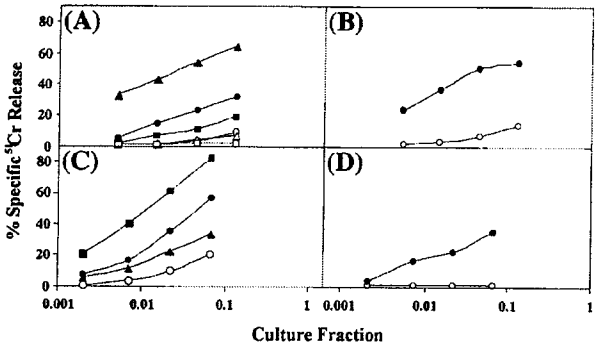

FIG. 8. Cytolytic T cells from peripheral blood of four individual rhesus macaques immunized with pCMVKm2.GagMod.SF2. PBMC were isolated 2 weeks after the second immunization (A and B) or 2 weeks after the third immunization (C and D). PBMC were cultured for 8 days in the presence of pools of synthetic Gag peptides (A and B) or with rVV*gag-pol*-infected PBMC (C and D). PBMC cultures were harvested and serially diluted as described in Materials and Methods, and Gag-specific cytolytic activity was assayed using autologous B-LCL target cells that had been pulsed with Gag peptide pools. (A) PBMC from rhesus macaque 63 stimulated with pool 1 and assayed on targets pulsed with pool 1 (●) or pool 5 (○), stimulated with pool 4 and assayed on targets pulsed with pool 4 (▲) or pool 8 (△), and stimulated with pool 5 and assayed on targets pulsed with pool 5 (■) or pool 1 (□); (B) PBMC from rhesus macaque 68 stimulated with pool 4 and assayed on targets pulsed with pool 4 (●) or pool 8 (○); (C) PBMC from rhesus macaque 77 stimulated with rVV*gag-pol* and assayed on targets pulsed with pool 1 (■), pool 5 (▲), pool 8 (●), or an Env peptide pool (○); (D) PBMC from rhesus macaque 78 stimulated with rVV*gag-pol* and assayed on targets pulsed with pool 2 (●) or an Env peptide pool (○).

had a very low titer 2 weeks after the second immunization (65), which later dropped below the detection level. These results reflect the induction of robust and relatively broad CTL responses using the modified *gag* plasmid following DNA immunization of nonhuman primates and warrant further study with these plasmids. This contrasts with previous results in which weak and transient CTL responses were observed in only one of four macaques given seven immunizations with 1-mg doses of the pCMVLink.Gag.SF2.PRE plasmid containing the native HIV-1_{SF2} *gag* (X. Paliard and C. Walker, unpublished data).

DISCUSSION

To increase the potency of HIV-1 DNA vaccines, we modified the genes coding for HIV-1_{SF2} Gag and Protease to overcome Rev dependence and to increase expression levels. Changes in codon usage to that utilized by highly expressed human genes in combination with inactivation of INS regions dramatically increased Gag expression from these constructs in the absence of Rev. Expression levels from the modified *gag* plasmid pCMVKm2.GagMod.SF2 were increased between 322- and 966-fold in 293 cells compared with those from pCMVLink.Gag.SF2.PRE, which contained the native HIV-1_{SF2} *gag* gene. Density gradient and electron microscopy analysis demonstrated that the modified *gag* genes efficiently expressed particles with the density and morphology expected for HIV VLP (Fig. 2 and 3). Similarly modified *gagprotease* plasmids that also showed high levels of Rev-independent expression were constructed (Table 2), but the expression cassette in which the codons for protease were optimized in combination with INS inactivation showed evidence of protease overexpression and reduced formation of VLP in transfected COS-7 cells (GP1; Fig. 4B). In contrast, both immature and mature VLP were produced from *gagprotease* constructs in which the INS were inactivated without codon optimization (GP2; Fig. 3 and 4).

In light of the improved expression levels from the modified *gag*, mouse studies were conducted to evaluate immune responses to this construct when administered as a DNA vaccine. When the modified *gag* plasmid was employed, Gag antigen-specific IFN-γ-secreting $CD8^+$ T cells could be measured following a single immunization with as little as 2 ng of plasmid DNA (Fig. 7). CTL responses were observed in a lysis assay after a single immunization with 20 ng of the modified *gag* plasmid. These results combined indicate a 10- to 100-fold improvement over the native *gag* plasmid, for which at least 200 ng of DNA was required for the induction of a detectable antigen-specific CTL response (Fig. 6). The improved potency of the modified *gag* was also reflected in the humoral responses. A single dose of 200 ng of the modified *gag* was sufficient to induce measurable anti-Gag antibody responses in 25% of the mice (Fig. 1A), while 100-fold more (20 μg) of the native *gag* plasmid was required for the detection of Gag-specific antibodies.

The improved potency of the codon-modified *gag* expression plasmid observed in mouse studies was confirmed with rhesus macaques. Four of four macaques had detectable Gag-specific CTL after two or three 1-mg doses of modified *gag* plasmid. In contrast, in a previous study, only one of four macaques given 1-mg doses of plasmid DNA encoding the wild-type HIV-1_{SF2} Gag showed strong CTL activity, which was not apparent until after the seventh immunization (X. Paliard and C. Walker, unpublished data). Further evidence of the potency of the modified *gag* plasmid was the observation that CTL from two of the four rhesus macaques reacted with three nonoverlapping Gag peptide pools, suggesting that as many as three different Gag peptides are recognized and indicating that the CTL response is polyclonal. Additional quantification and specificity studies are in progress to further characterize the T-cell responses to Gag in plasmid-immunized rhesus macaques. DNA immunization of macaques with the modified *gag* plasmid did not result in significant antibody responses, with only two of four animals seroconverting at low titers. In contrast, the majority of macaques in additional groups immunized with $p55^{Gag}$ protein seroconverted and had strong Gag-specific antibody titers (G. Otten, unpublished data). These preliminary data together with data from other investigators indicate that a prime-boost strategy, with DNA prime and protein boost, could be very promising for the induction of strong CTL and antibody responses.

These results indicate that sequence-modified high-level expression cassettes for HIV structural genes can improve the potency of plasmid-vectored HIV vaccines. Sequence-modified genes may also enhance the potency of virus-vectored vaccines and increase the production efficiency of HIV structural proteins for use in subunit vaccines.

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TAB 4

Masschalck *et al.*, "Inactivation of gram-negative bacteria by lysozyme, denatured lysozyme, and lysozyme-derived peptides under high hydrostatic pressure," Appl Environ Microbiol. 2001 Jan;67(1):339-44

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Inactivation of Gram-Negative Bacteria by Lysozyme, Denatured Lysozyme, and Lysozyme-Derived Peptides under High Hydrostatic Pressure

BARBARA MASSCHALCK, ROB VAN HOUDT, ELLEN G. R. VAN HAVER, AND CHRIS W. MICHIELS*

Laboratory of Food Microbiology, Katholieke Universiteit Leuven, B-3001 Heverlee, Belgium

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We have studied the inactivation of six gram-negative bacteria (*Escherichia coli*, *Pseudomonas fluorescens*, *Salmonella enterica* serovar Typhimurium, *Salmonella enteritidis*, *Shigella sonnei*, and *Shigella flexneri*) by high hydrostatic pressure treatment in the presence of hen egg-white lysozyme, partially or completely denatured lysozyme, or a synthetic cationic peptide derived from either hen egg white or coliphage T4 lysozyme. None of these compounds had a bactericidal or bacteriostatic effect on any of the tested bacteria at atmospheric pressure. Under high pressure, all bacteria except both *Salmonella* species showed higher inactivation in the presence of 100 μg of lysozyme/ml than without this additive, indicating that pressure sensitized the bacteria to lysozyme. This extra inactivation by lysozyme was accompanied by the formation of spheroplasts. Complete knockout of the muramidase enzymatic activity of lysozyme by heat treatment fully eliminated its bactericidal effect under pressure, but partially denatured lysozyme was still active against some bacteria. Contrary to some recent reports, these results indicate that enzymatic activity is indispensable for the antimicrobial activity of lysozyme. However, partial heat denaturation extended the activity spectrum of lysozyme under pressure to serovar Typhimurium, suggesting enhanced uptake of partially denatured lysozyme through the serovar Typhimurium outer membrane. All test bacteria were sensitized by high pressure to a peptide corresponding to amino acid residues 96 to 116 of hen egg white, and all except *E. coli* and *P. fluorescens* were sensitized by high pressure to a peptide corresponding to amino acid residues 143 to 155 of T4 lysozyme. Since they are not enzymatically active, these peptides probably have a different mechanism of action than all lysozyme polypeptides.

High hydrostatic pressure treatment is a promising technique for cold pasteurization of foods that allows better retention of product flavor, texture, color, and nutrient content than a comparable conventional heat pasteurization (17, 24). The main obstacles that prevent a commercial breakthrough of pressure-preserved foods are the high investment cost, due to the high pressures required for efficient microbe and enzyme destruction, and the paucity of knowledge on the sensitivity of various pathogenic and spoilage microorganisms to hydrostatic pressure and on the factors affecting this sensitivity.

The application of hurdle technology has been proposed as an approach to increase the microbicidal effect of the process at lower pressures. Hurdle technology relies on the synergistic combination of moderate doses of two or more microbe-inactivating and/or growth-retarding factors (18). An interesting example of synergistic inactivation exists between high pressure and a number of antimicrobial peptides, including nisin, lysozyme, and pediocin (3, 9, 14, 19). This synergistic inactivation was observed not only in intrinsically sensitive gram-positive bacteria but also in gram-negative bacteria, which are normally insensitive to these peptides because their cellular targets are shielded by their outer membrane.

In the present work we have focused on lysozyme because it has some interesting features for application as a food preservative. First, lysozymes are naturally present in foods such as egg white (ca. 3.2 mg/ml) (1), cow milk (ca. 0.13 μg/ml) (1), and human colostrum (ca. 65 μg/ml) (20) but also in several plants, such as cauliflower (ca. 27.6 μg/ml) and cabbage (ca. 2.3 μg/ml) (22). The use of these naturally occurring lysozymes at a concentration of 10 to 100 μg/ml, as proposed in this work, should therefore not present a toxicological concern. Second, the bacteriostatic and bactericidal properties of lysozyme have been the subject of many studies, and over the last 10 years, several authors have proposed a novel antibacterial mechanism of action for lysozyme that is independent of its 1,4-β-*N*-acetylmuramidase activity. These reports are based on unique bactericidal properties observed with partially or completely denatured lysozymes having reduced or no enzymatic activity against both gram-positive and gram-negative bacteria (2, 10, 13, 16). For example, Ibrahim et al. (10, 13) demonstrated inactivation by heat-denatured lysozyme of an *Escherichia coli* K-12 strain, which was relatively insensitive to native lysozyme. Düring et al. (2) showed that native and heat-treated enzymatically inactive lysozyme from coliphage T4 caused similar inactivation levels on a particular *E. coli* strain. The antibacterial properties of denatured lysozyme have been proposed to result from the cationic nature of the peptide in combination with conformational changes leading to increased hydrophobicity. These characteristics are believed to contribute to the antimicrobial properties of several peptides (8). Specific peptides with cationic properties and without enzymatic activity derived from hen egg white and T4 lysozyme were also found to have antimicrobial activity (2, 21).

* Corresponding author. Mailing address: Laboratory of Food Microbiology, Katholieke Universiteit Leuven, Kard. Mercierlaan 92, B-3001 Heverlee, Belgium. Phone: 32-16-321578. Fax: 32-16-321960. E-mail: Chris.michiels@agr.kuleuven.ac.be.

The present work extends our previous observation that high pressure sensitizes *E. coli* for lysozyme (3, 9, 19). We have studied the effect of denatured lysozymes and lysozyme-derived peptides, in addition to native lysozyme, in combination with high pressure on a panel of six different gram-negative bacteria including several foodborne pathogens. The results of this work should provide better insight into the mode of antibacterial action of lysozyme and contribute to the development of more efficient technology for cold high-pressure pasteurization.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. The bacteria used in this work are *E. coli* K-12 strain MG1655 (5), *Pseudomonas fluorescens* LMMBM07, *Salmonella enterica* serovar Typhimurium LMMBM01 (both from our laboratory collection), *Salmonella enteritidis* ATCC13076 (from the American Type Culture Collection), *Shigella flexneri* LMG10472 and *Shigella sonnei* LMG10473 (both from the Belgian Coordinated Culture Collection of Microorganisms, Ghent, Belgium). All experiments were carried out with cultures in stationary phase, obtained by growth in nutrient bouillon (Oxoid, Basingstoke, United Kingdom) for 21 h with shaking (200 rpm) at 37°C, except for *P. fluorescens*, which was incubated at 30°C.

Growth inhibition. Growth inhibition by lysozymes and lysozyme-derived peptides was determined by recording growth curves in triplicate with a Bioscreen C microbiology reader (Labsystems Oy, Helsinki, Finland). Stationary phase cultures were diluted to between 5×10^3 and 5×10^4 cells/ml in fresh medium, and 360 μl was transferred to the honeycomb plate wells of the Bioscreen C reader. The volume was then adjusted to 400 μl, either with buffer for the controls or with the appropriate solution of antimicrobial. Every 15 min the cultures were shaken at medium intensity for 1 min, and the turbidity was measured with a wide band filter. The growth curves were followed for 30 h, in which time all bacteria reached the stationary phase.

Denaturation of lysozyme and measurement of lysozyme enzymatic activity. Hen egg-white lysozyme (66,000 U/mg; Fluka, Buchs, Switzerland) was stored frozen (−20°C) as a stock solution of 1 mg/ml in potassium phosphate buffer (10 mM, pH 7.0). For heat denaturation, 100 μl of the 1-mg/ml solution was transferred to a sterile glass capillary and treated for 20 or 60 min at 80 or 100°C in a water bath. For denaturation with β-mercapto-ethanol, the 1-mg/ml lysozyme solution was incubated with 5% β-mercapto-ethanol during 1 h at 60°C. After the treatments, the lysozyme samples were put directly on ice and stored at −20°C.

The enzymatic activity of lysozyme and its derivatives was measured with lyophilized *Micrococcus lysodeikticus* (ATCC 4698) cells (Fluka) resuspended at 0.5 mg/ml in 10 mM potassium phosphate buffer (pH 7.0) as a substrate using a method adapted from Wolsnor (25). Thirty-microliter aliquots of different dilutions of the sample were added to 300 μl of *M. lysodeikticus* cell suspension, and the lysis of cells was measured automatically as the decrease in turbidity (optical density at 600 nm [OD_{600}]) with the Bioscreen C microbiology reader during 40 min at 20°C. The dilution resulting in a rate of turbidity decrease between 0.001 and 0.005 OD U/min was used to calculate the enzymatic activity. Enzymatic activity was expressed in units per milligram of protein or as a percentage relative to untreated lysozyme.

Synthetic peptides. Two synthetic peptides (95% purity) were purchased from Eurogentec (Herstal, Belgium). Peptide HEL96-116 (H_2N-KKI VSD GNG MNA WVA WRK RCK-COOH) is a 21-mer peptide corresponding to amino acids 96 to 116 of hen egg-white lysozyme (HEL). Peptide T4L143-155 (H_2N-PNR AKR VIT TFR T-COOH) is a 13-mer peptide and corresponds to amino acids 143 to 155 of bacteriophage T4 lysozyme (T4L).

Pressure treatment. Cells in stationary phase were harvested by centrifugation (3,800 × *g*, 5 min) and resuspended in the same volume of potassium phosphate buffer (10 mM, pH 7.0), yielding a final cell population of 5×10^8 to 5×10^9 CFU/ml. Although the pH of phosphate buffer is more pressure dependent than the pH of some other buffers (15), phosphate buffer was chosen for this study because it is widely used in inactivation studies and is not harmful to microorganisms. After the addition of lysozyme or one of its derivatives where appropriate, cell suspensions (300 μl) were sealed without air bubbles in sterile polyethylene bags and subjected at 20°C to pressures in the range of 155 to 300 MPa. Pressure treatment was done in a system with eight parallel thermostatically controlled 8-ml vessels which could be simultaneously pressurized and individually decompressed at different times (Resato, Roden, The Netherlands). The compression rate was approximately 100 MPa/min; decompression was in less than 3 s. The high-pressure transmission fluid used was Resato high-pressure fluid TP1, a mixture of glycols (Van Meeuwen, Wesp, The Netherlands). The pressurization times reported do not include the come-up and come-down time. It should also be noted that the temperature in the vessels could not be kept constant, due to adiabatic compression and decompression. Temperature measurements with thermocouples inside the pressure vessels, which had been previously conducted under identical circumstances, suggested a temperature increase to 29°C upon rapid pressurization to 300 MPa.

To measure bactericidal activities at atmospheric pressure, a part of the suspension with the additives was kept at room temperature without pressurization and was plated after the same exposure time as the pressurized samples.

Enumeration of viable cells. Appropriate dilutions in sterile potassium phosphate buffer (10 mM, pH 7.0) were surface plated with a spiral plater (Spiral System Inc., Cincinnati, Ohio) on tryptic soy agar for *E. coli* and *P. fluorescens* or on plate count agar (both media from Oxoid) for the other bacteria. The plated volume was 50 μl, and hence the detection limit was 20 CFU/ml. Colonies were allowed to develop for 24 to 48 h at the appropriate incubation temperature. Inactivation was expressed as a logarithmic viability reduction, log (N_0/N), with N and N_0 the colony counts after a treatment and in the untreated sample, respectively. For all treatments, averages ± standard deviations for at least three independent cultures of each strain are shown. Significant differences were calculated with the paired Student's *t* test.

RESULTS

Sensitization for native lysozyme. The test panel of six gram-negative bacteria including four pathogens (two *Salmonella* and two *Shigella* strains) was screened for sensitivity to native lysozyme under conditions of ambient and of elevated pressure. Two concentrations, 10 and 100 μg of lysozyme/ml, were used to investigate dose dependency. Growth curves in the presence of lysozyme revealed no inactivation or growth retardation for any of the bacteria, even at 100 μg of lysozyme/ml (data not shown). Higher concentrations of lysozyme were not tested, since these tended to cause aggregation of the bacteria, making the plate counts unreliable.

Sensitization for lysozyme by high pressure was tested by adding lysozyme to the bacterial suspensions before pressure treatment (Table 1). For each strain a pressure was chosen that, in the absence of lysozyme, caused an inactivation of at least 1 log unit. We speculated that in this way the pressure treatment would be severe enough to sensitize the cells to lysozyme. Because of their different pressure sensitivities, a uniform pressure treatment could not be used for all the bacteria. At 10 μg/ml, only *E. coli* and *P. fluorescens* were sensitized to lysozyme by high pressure, but at 100 μg/ml, *S. flexneri* and *S. sonnei* also became sensitive, and the extra logarithmic viability reduction caused by lysozyme for *E. coli* and *P. fluorescens* increased from 0.4 to 1.0 and from 1.5 to 2.5, respectively. Neither *Salmonella* strain was inactivated by lysozyme under pressure, and the presence of 100 μg of lysozyme/ml even had a protective effect against pressure inactivation for *Salmonella* serovar Typhimurium. In three treatments (*S. sonnei* with lysozyme at 10 and 100 μg/ml, and serovar Typhimurium at 100 μg/ml), standard deviations were remarkably higher than in any other treatment. These experiments were repeated in triplicate but the standard deviations remained high.

Sensitivity for lysozyme under pressure was transient, since exposure of pressure-treated cells to lysozyme (1 h at room temperature) after pressure treatment did not cause further inactivation, not even for *P. fluorescens*, which was most sensitive for lysozyme under pressure.

Aug-28-09 11:29am From-Novartis V&D IP Dept 510-923-3542 T-207 P.024/030 F-510

TABLE 1. Logarithmic viability reduction of bacterial suspensions

Organism	Pressure (MPa)	Log $N_0/N \pm SD^a$ of suspensions with:				No. of samples
		No additives (control)	Lys added[b]			
			10 μg/ml	100 μg/ml	10 μg/ml, after pressure[c]	
E. coli	300	1.6 ± 0.1 (a)	2.0 ± 0.1 (b)	2.6 ± 0.4 (b)	1.8 ± 0.2 (a)	3
P. fluorescens	155	1.6 ± 0.5 (a)	3.1 ± 0.3 (b)	4.1 ± 0.3 (c)	1.7 ± 0.1 (a)	3
S. enteritidis	270	1.9 ± 0.3 (a)	2.0 ± 0.0 (a)	1.9 ± 0.2 (a)	2.0 ± 0.1 (a)	3
Salmonella serovar Typhimurium	250	1.6 ± 0.4 (a)	1.4 ± 0.4 (a)	1.2 ± 0.9 (b)	1.5 ± 0.3 (a)	6
S. flexneri	250	3.5 ± 0.3 (a)	3.7 ± 0.4 (a)	4.6 ± 0.2 (b)	3.5 ± 0.4 (a)	3
S. sonnei	220	4.3 ± 0.3 (a)	3.8 ± 1.0 (a)	5.6 ± 1.6 (b)	4.2 ± 0.3 (a)	6

[a] Values within a row followed by different letters are significantly different ($P < 0.05$).
[b] Lys, native hen egg-white lysozyme.
[c] Pressure treatment: 15 min, 20°C, 10 mM phosphate buffer, pH 7.0.

Sensitization for partially heat- or β-mercapto-ethanol-denatured lysozyme. It has been suggested by some authors that partial or even complete heat denaturation would extend the working spectrum of lysozyme to some gram-negative bacteria that are not normally sensitive to lysozyme (2, 10, 13). We wanted to confirm these remarkable observations on our own panel of test bacteria and to compare the bactericidal efficiency of native and denatured lysozymes under high pressure. First, we tested whether lysozyme is denatured by the high pressure treatment itself. This seems not to be the case, since even after a harsh treatment at 600 MPa and 60°C during 15 min, 100% of the enzymatic activity was retained (Table 2). Lysozyme was denatured with heat in a way similar to that described by Ibrahim et al. (10, 13) and, in addition, with β-mercapto-ethanol. The different treatments of lysozyme, the designations used for each denatured product, and the corresponding remaining enzymatic activities are shown in Table 2. Two denatured forms (H100/20-lys and M-lys) were almost completely enzymatically inactive with, respectively, 0.6 and 0.5% of residual activity whereas one (H80/20-lys) still had 11.5% of lytic activity. In the high pressure experiments, H80/20-lys was applied at 10 μg/ml and the more denatured forms at 10 and 100 μg/ml.

In control experiments at atmospheric pressure, no bacteriostatic or bactericidal activity could be detected for any of the denatured forms on any of the test bacteria (data not shown). Since the solution of M-lys still contained β-mercapto-ethanol, another series of control experiments was performed in which all test bacteria were pressure treated in the presence of 5% β-mercapto-ethanol, but no enhanced lethality was observed due to the presence of this compound (data not shown). Table 3 shows the reduction factors of all the test bacteria when treated with the denatured lysozymes under high pressure. In general, denatured lysozymes were active under pressure against fewer bacteria than intact lysozyme at the same concentration. Both strongly denatured lysozymes (H100/20-lys and M-lys) were completely inactive under pressure at 10 μg/ml, although they remained active against at least one of the bacteria at 100 μg/ml. An interesting observation concerns serovar Typhimurium, which was sensitive under pressure for some of the denatured lysozymes (H80/20-lys at 10 μg/ml and H100/20-lys at 100 μg/ml), while it was not sensitive for native lysozyme even at 100 μg/ml. This remarkable behavior of serovar Typhimurium was confirmed in an experiment with six replicate samples. Serovar Typhimurium was not sensitive, however, to the β-mercapto-ethanol-denatured lysozyme (M-lys), although the latter had almost the same level of enzymatic activity as H100/20-lys. M-lys at 100 μg/ml was active only against *P. fluorescens*. This organism was also sensitive to H80/20-lys (10 μg/ml) and H100/20-lys (100 μg/ml) under pressure and was therefore the most sensitive of the tested bacteria.

Contribution of enzymatic activity to the bactericidal properties of lysozymes under pressure. In the experiment described above, the denatured lysozymes always had some residual enzymatic activity. To clarify whether enzymatic activity is necessary at all for the bactericidal effects observed with denatured lysozymes under pressure, we subjected lysozyme to heat treatment at 100°C during 60 min, obtaining a sample with undetectable enzymatic activity (H100/60-lys). The inactivation of *P. fluorescens*, as the most sensitive of all tested bacteria in the previous experiments, by H100/60-lys and H80/20-lys under pressure was subsequently compared and it was found that the completely denatured lysozyme had lost its bactericidal activity (data not shown). A further confirmation for the role of peptidoglycanolytic activity was found by microscopic observation of cell morphology after pressure treatment. The bactericidal effect caused by intact or partially denatured lysozyme under high pressure was always accompanied by a change in the morphology of the cells from rod to sphere. This change did not occur in the absence of lysozyme or with the completely denatured lysozyme and is therefore most likely due to the formation of spheroplasts as a result of the residual lytic activity of lysozyme.

TABLE 2. Residual enzymatic activity of partially denatured hen egg-white lysozyme solutions[a]

Designation	Treatment of Lys	Residual enzymatic activity	
		% of untreated Lys	U/mg
Lys	No treatment	100.0	66,000
Pressurized Lys	60°C, 15 min, 600 MPa	100.0	66,000
H80/20-lys	80°C, 20 min	11.5	7,590
H100/20-lys	100°C, 20 min	0.6	396
M-lys	5% β-mercapto-ethanol, 60°C, 60 min	0.5	330

[a] Lys, lysozyme.

TABLE 3. Logarithmic viability reduction of bacteria by denatured hen egg-white lysozymes under high pressure[a]

Organism	Pressure (MPa)	Log $N_0/N \pm$ SD[b] of bacteria with:						No. of samples
		No additives (control)[c]	H80/20-lys (10 μg/ml)	H100/20-lys		M-lys		
				10 μg/ml	100 μg/ml	10 μg/ml	100 μg/ml	
E. coli	300	1.6 ± 0.1 (a)	1.9 ± 0.1 (a)	1.4 ± 0.2 (a)	1.9 ± 0.1 (a)	1.5 ± 0.2 (a)	1.9 ± 0.1 (a)	3
P. fluorescens	155	1.6 ± 0.5 (a)	3.1 ± 0.1 (c)	1.7 ± 0.2 (a)	2.5 ± 0.1 (b)	1.6 ± 0.1 (a)	2.6 ± 0.1 (b)	3
S. enteritidis	270	1.9 ± 0.3 (a)	2.0 ± 0.3 (a)	1.7 ± 0.2 (a)	2.3 ± 0.2 (a)	1.7 ± 0.2 (a)	1.9 ± 0.3 (a)	3
Salmonella serovar Typhimurium	250	1.6 ± 0.4 (a)	2.1 ± 0.3 (b)	1.6 ± 0.2 (a)	2.2 ± 0.1 (b)	1.6 ± 0.3 (a)	1.7 ± 0.2 (a)	6
S. flexneri	250	3.5 ± 0.3 (a)	3.3 ± 0.2 (a)	3.2 ± 0.2 (a)	3.8 ± 0.5 (a)	3.2 ± 0.3 (a)	4.0 ± 0.5 (a)	3
S. sonnei	220	4.3 ± 0.3 (a)	4.0 ± 0.2 (a)	3.8 ± 0.4 (a)	4.4 ± 0.4 (a)	3.8 ± 0.4 (a)	5.1 ± 0.9 (a)	3

[a] High pressure treatment: 15 min, 20°C, 10 mM phosphate buffer, pH 7.0.
[b] Values within a row followed by different letters are significantly different ($P < 0.05$).
[c] Results in this table and in Table 1 were obtained with the same bacterial cultures. Therefore, the values shown for the control treatment are taken from Table 1.

Sensitization for lysozyme-derived peptides. In a final set of experiments, two synthetic peptides derived, respectively, from hen egg-white lysozyme (HEL96-116) and *E. coli* bacteriophage T4 lysozyme (T4L143-155) were investigated for antibacterial activity. Peptide HEL96-116 is similar to the bactericidal peptide of 15 amino acids that was isolated by Pellegrini et al. (21) by digesting lysozyme with the protease clostripain but has two additional NH_2-terminal and four additional COOH-terminal amino acids from the original lysozyme sequence. This increases the cationic character of the peptide, which is known to contribute to the antibacterial activity of several antibiotic peptides (7). Peptide T4L143-155 was chosen and synthesized by Düring et al. (2) for its amphiphatic character and helicoidal structure and was also found to have bactericidal activity. The calculated isoelectric points of HEL96-116 and T4L143-155 are 10.29 and 12.40, respectively, and both peptides were completely enzymatically inactive.

These two peptides were applied to our test panel of bacteria at 100 μg/ml. At atmospheric pressure neither growth inhibition nor inactivation was observed (data not shown). Under pressure (Table 4), all bacteria were sensitized for the HEL96-116 peptide, even serovar Typhimurium and *S. enteritidis*, which were not sensitized for native lysozyme under pressure. On the other hand, peptide T4L143-155 was active under pressure against all bacteria except *E. coli* and *P. fluorescens*, two bacteria that were very sensitive to native lysozyme under pressure. HEL96-116 was more effective than T4L143-155 against all bacteria, and bactericidal activity of both peptides under pressure was not accompanied by spheroplast formation.

DISCUSSION

A first objective of this work was to investigate whether the previously reported observation that high pressure can sensitize *E. coli* to lysozyme (3, 9, 19) can be extended to other gram-negative bacteria. It was found that this is the case for some but not for all gram-negative bacteria (Table 1). Inactivation by lysozyme under pressure was concentration dependent since at 100 μg/ml more bacteria were sensitized and a higher magnitude of sensitization occurred than at 10 μg/ml. In line with what was reported earlier by Hauben et al. (9) for *E. coli*, we found that sensitization is transient. As soon as pressure was released, all bacteria immediately regained their resistance to lysozyme. In addition to this type of transient sensitization, high pressure also causes a persistant type of sensitization, for instance to the lactoperoxidase system (4). Of course, whether or not an organism gets sensitized to lysozyme by high pressure may depend on many other factors, such as pressure, temperature, pH, medium composition, and cell growth stage and history. For example, we have previously demonstrated that application of pressure pulses with brief interruptions can cause sensitization to lysozyme and nisin of bacteria that are not sensitized by a continuous pressure treatment (19).

In the present work, we explored another route to maximize the synergistic bactericidal effect of pressure and lysozyme, by replacing native lysozyme with denatured forms of lysozyme and peptides derived from lysozyme, to both of which have been ascribed certain antimicrobial effects previously.

We prepared heat-denatured lysozyme according to Ibrahim

TABLE 4. Logarithmic viability reduction of bacterial suspensions treated with high pressure[a] both without additives and with the addition of lysozyme-derived peptides

Organism	Pressure (MPa)	Log $N_0/N \pm$ SD[b] with:			No. of samples
		No additives (control)	HEL96-116 (100 μg/ml)	T4L143-155 (100 μg/ml)	
E. coli	300	3.2 ± 0.5 (a)	4.4 ± 0.2 (b)	3.5 ± 0.2 (a)	3
P. fluorescens	155	1.0 ± 0.1 (a)	2.1 ± 0.4 (b)	1.3 ± 0.7 (a)	3
S. enteritidis	270	3.2 ± 0.2 (a)	4.4 ± 0.2 (b)	4.3 ± 0.2 (b)	3
Salmonella serovar Typhimurium	250	2.2 ± 1.3 (a)	3.2 ± 0.8 (b)	3.0 ± 1.2 (b)	6
S. flexneri	250	0.9 ± 0.3 (a)	2.5 ± 0.4 (b)	2.3 ± 0.1 (b)	3
S. sonnei	220	1.7 ± 1.0 (a)	3.4 ± 1.1 (b)	2.9 ± 0.9 (b)	6

[a] High pressure treatment: 15 min, 20°C, 10 mM phosphate buffer, pH 7.0.
[b] Values within a row followed by different letters are significantly different ($P < 0.05$).

Aug-28-09 11:30am From-Novartis V&D IP Dept 510-923-3542 T-207 P.026/030 F-510

et al. (10, 13), but we failed to confirm any of the bactericidal or bacteriostatic effects under atmospheric pressure that were reported by these authors. Denaturation with β-mercapto-ethanol also did not endow lysozyme with antimicrobial activity at atmospheric pressure. We believe therefore that the effects described are very strain dependent.

The experiments with the denatured lysozymes under high pressure (Tables 2 and 3) lead to two observations. One is that the enzymatic activity of lysozyme is required for it to exert a bactericidal effect under pressure. Reduction of enzymatic activity by heat or β-mercapto-ethanol denaturation clearly leads to a reduction in the observed bactericidal effect, and complete elimination of enzymatic activity by extended heat treatment (60 min, 100°C) completely eliminates the bactericidal effect. At this point, therefore, our results do not allow us to confirm the hypothesis raised by other authors that the antimicrobial activity of lysozyme and/or heat-denatured lysozyme would consist partly of a mechanism that is independent of enzymatic activity (10, 13, 21). The second observation from these experiments is that partial heat denaturation can extend the spectrum of lysozyme bactericidal activity under pressure to a wider range of bacteria. In our experiments, heat denaturation made lysozyme active against serovar Typhimurium. A threshold level of residual enzymatic activity remains a requirement also in this case, as can be deduced from the results in Table 3. The mildly denatured lysozyme H80/20-lys (with 11.5% residual activity) is active against serovar Typhimurium at 10 μg/ml under pressure, while the extensively denatured H100/20-lys (with only 0.6% residual activity) is active only at 100 μg/ml.

Taken together, these results allow us to formulate the following hypothesis about the synergistic effect of high pressure and lysozyme on the inactivation of gram-negative bacteria. At ambient pressure, lysozyme is completely inactive against most gram-negative bacteria because it cannot penetrate the outer membrane to reach its target, the peptidoglycan. Nevertheless, lysozyme has both cationic and lipophilic properties, which are known to contribute to an intimate interaction with and passage through bilayer membranes of many small peptides with antibacterial properties (8). Passage is believed to occur through the so-called self-promoted uptake mechanism (6). Interestingly, it has been demonstrated that deep rough mutants of serovar Typhimurium are sensitive to lysozyme (23), suggesting that small changes in outer membrane composition may allow self-promoted uptake of lysozyme. Conversely, a change in the structure of lysozyme by heat denaturation can also cause the enzyme to become active against strains of *E. coli* which are insensitive to native lysozyme (10, 13). Under high pressure, the ultimate mode of action of lysozyme remains the same, i.e., the peptidoglycanolytic activity. However, pressure apparently stimulates passage of lysozyme through the outer membrane of several gram-negative bacteria. We have previously named this phenomenon pressure-assisted self-promoted uptake, or, briefly, pressure-promoted uptake (19). Our present results show that pressure-promoted uptake of lysozyme is not a universal phenomenon in all gram-negative bacteria, probably because it depends on subtle properties of, and interactions between, the cell surface and the lysozyme molecule. Subtle changes in either the cell surface or lysozyme structure may change the outcome of the interaction. For example, an *E. coli* mutant has been decribed that is resistant to lysozyme under pressure (19). In the present work, mild denaturation of lysozyme allowed successful pressure-promoted uptake and bactericidal action against serovar Typhimurium, which is refractory to pressure-promoted uptake of native lysozyme. The underlying explanation may be an increase in the hydrophobicity of lysozyme by heat denaturation, since it has been shown previously that increasing lysozyme hydrophobicity by genetic fusion of a hydrophobic peptide to the NH_2 terminal of the enzyme (12) or by chemical modification of the lysyl residues with saturated fatty acids (11) enhanced the activity of lysozyme against gram-negative bacteria. An alternative mode of antibacterial action that has been proposed for lysozyme is that the binding of lysozyme to the bacterial envelope would activate the autolysins (13, 16). As a second part of this work, the bactericidal effect under pressure of two specific lysozyme-derived peptides to which antibacterial properties had been previously assigned (2, 21) was investigated. Again, we were unable to reproduce these effects with our panel of test bacteria and under our experimental conditions. However, both peptides were very effective under high pressure, in particular the peptide derived from hen egg white, which was active against all test bacteria (Table 4). For these peptides, a different mechanism of bactericidal action must be involved because they are completely devoid of enzymatic activity. The precise mechanism remains unknown, but it seems evident that, similar to lysozyme, bactericidal activity will depend on passage through the outer membrane.

The use of lysozyme, heat-denatured lysozyme, and peptides derived from lysozyme may find interesting applications in the nonthermal preservation of foods and pharmaceutical and other products by high pressure. In the presence of these additives, lower pressures can be used to achieve the desired reduction factors, making high hydrostatic pressure technology more economically feasible. For optimal performance, the working spectrum of these compounds under pressure will have to be studied in more detail, and it may be necessary to design mixtures of specific compounds to cover as wide a range of bacteria as possible. For the peptides derived from lysozyme, toxicity studies should be conducted to demonstrate their safety before they can be applied in foods.

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L14 ANSWER 1 OF 9 BIOSIS COPYRIGHT (c) 2009 The Thomson Corporation on STN
AN 1982:296084 BIOSIS
DN PREV198274068564; BA74:68564
TI AN ORAL ENTERITIS VACCINE COMPOSED OF 12 HEAT INACTIVATED ENTEROBACTERIACEAE 3. STUDIES ON EFFICACY TESTS IN MICE PROTECTION TESTS.
AU RAETTIG H [Reprint author]
CS SENHEIMERSTR 45 A, D-1000 BERLIN 28 FROHNAU
SO Zentralblatt fuer Bakteriologie Mikrobiologie und Hygiene 1 Abt Originale A, (1981) Vol. 205, No. 4, pp. 511-520.
CODEN: ZBMPDI. ISSN: 0174-3031.
DT Article
FS BA
LA GERMAN
AB A polyvalent vaccine composed of 12 heat-inactivated Enterobacteriaceae spp. (including Shigella spp., Salmonella spp. and Escherichia coli strains) was studied in mice. The success of vaccination was reported. The efficacy of the oral immunization depended on the dosage of the vaccine. Storage of this vaccine was discussed.

Chang *et al.*, "UV inactivation of pathogenic and indicator microorganisms," Appl Environ Microbiol. 1985 Jun;49(6):1361-5, abstract

L5 ANSWER 10 OF 23 MEDLINE on STN
AN 1985250400 MEDLINE
DN PubMed ID: 2990336
TI UV inactivation of pathogenic and indicator microorganisms.
AU Chang J C; Ossoff S F; Lobe D C; Dorfman M H; Dumais C M; Qualls R G; Johnson J D
SO Applied and environmental microbiology, (1985 Jun) Vol. 49, No. 6, pp. 1361-5.
Journal code: 7605801. ISSN: 0099-2240.
Report No.: NLM-PMC241729.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, U.S. GOV'T, NON-P.H.S.)
LA English
FS Priority Journals
EM 198508
ED Entered STN: 20 Mar 1990
Last Updated on STN: 20 Mar 1990
Entered Medline: 5 Aug 1985
AB Survival was measured as a function of the dose of germicidal UV light for the bacteria Escherichia coli, Salmonella typhi, Shigella sonnei, Streptococcus faecalis, Staphylococcus aureus, and Bacillus subtilis spores, the enteric viruses poliovirus type 1 and simian rotavirus SA11, the cysts of the protozoan Acanthamoeba castellanii, as well as for total coliforms and standard plate count microorganisms from secondary effluent. The doses of UV light necessary for a 99.9% inactivation of the cultured vegetative bacteria, total coliforms, and standard plate count microorganisms were comparable. However, the viruses, the bacterial spores, and the amoebic cysts required about 3 to 4 times, 9 times, and 15 times, respectively, the dose required for E. coli. These ratios covered a narrower relative dose range than that previously reported for chlorine disinfection of E. coli, viruses, spores, and cysts.

Kruithof *et al.*, "UV/H2O2-treatment: The ultimate solution for pesticide control and disinfection," Proceedings- Annual Conference, American Water Works assoc. 2000, p331-334, abstract

L13 ANSWER 12 OF 65 CAPLUS COPYRIGHT 2009 ACS on STN
AN 2001:707761 CAPLUS
DN 136:42400
TI UV/H2O2-treatment: The ultimate solution for pesticide control and disinfection
AU Kruithof, Joop C.; Kamp, Peer C.; Finch, Gordon R.
CS N.V. PWN Water Supply Company North Holland, Velserbroek, 1990 AC, Neth.
SO Proceedings - Annual Conference, American Water Works Association (2000) 331-344
CODEN: PWACDO; ISSN: 0360-814X
PB American Water Works Association
DT Journal; (computer optical disk)
LA English
AB High pesticide and microorganism content in IJssel Lake water compelled the N.V. PWN Water Supply Company North Holland (PWN) to implement multiple barriers against these pollutants at surface water treatment plants in Heemskerk and Andijk, Netherlands. For pesticide degradation, advanced oxidation was pursued. After thorough studies, O3/H2O2 treatment was rejected due to bromate formation. Subsequently UV/H2O2 treatment was pursued. UV-photolysis showed selective pesticide degradation conversion using 1 kWh/m3 elec. energy varied from 18% for trichloroacetic acid to 70% for atrazine. UV/H2O2 treatment resulted in much more selective pesticide degradation: with a combination of 1 kWh/m3 elec. energy and 15 g/m3 H2O2, most pesticides were degraded >80%, and formation of harmful byproducts was insignificant. At 0.1-2.5 kWh/m3 elec. energy and 0-25 g/m3 H2O2, bromate formation did not occur and metabolite formation was insignificant. Adsorbable organic C (AOC) formation was ≤140 μg/L; therefore, AOC and residual H2O2 must be removed in a subsequent treatment step. Granular activated C filtration showed very reliable results. Applying UV/H2O2 treatment for pesticide control with an elec. energy of 1 kWh/m3 goes together with an average UV-dose of 2000 mJ/cm2. This dose is .apprx.50 times as high as applied in conventional UV disinfection. As expected, Escherichia coli and (after spiking) sulfite-reducing Clostridia spores were completely inactivated. Orientating expts. were conducted with a UV dose of 100 mJ/cm2. MS-2 phage inactivation was 2.7 log, Bacillus spores inactivation was 3.4 log, and Cryptosporidium inactivation was complete (>3 log). Addnl. data were collected in cooperation with the University of Alberta. From these very promising results, application of UV/H2O2 treatment will be pursued for full scale application at the Heemskerk Plant for organic pollutant control and the Andijk Plant for organic pollutant control and disinfection.
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Sambrook *et al.*, Molecular Cloning: a Laboratory Manual 2nd ed, 1989. Vol 1, Page 1.3

1

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

J. Sambrook
UNIVERSITY OF TEXAS SOUTHWESTERN MEDICAL CENTER

E.F. Fritsch
GENETICS INSTITUTE

T. Maniatis
HARVARD UNIVERSITY

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1989

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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Essential Features of Plasmids

Replication and Incompatibility

Replication of plasmid DNA is carried out by subsets of enzymes used to duplicate the bacterial chromosome. However, different plasmids use different subsets and replicate to different extents in their hosts. Some reach copy numbers as high as 700 per cell, whereas others are maintained at the minimal level of 1 plasmid molecule per host-cell chromosome. The control of plasmid copy number resides in a region of the plasmid DNA that includes the origin of DNA replication. Usually, a plasmid will contain only one origin of replication together with its associated *cis*-acting control elements (the whole genetic unit being defined as a "replicon"). Very rarely, however, plasmids that have been generated by fusion will contain more than one replicon; in such cases, only one replicon is active.

Most vectors in current use carry a replicon derived from the plasmid pMB1, which was originally isolated from a clinical specimen (Hershfield et al. 1974). Under normal conditions of growth, a minimum of 15–20 copies of plasmids carrying this replicon (or its close relative the ColE1 replicon) (Bolivar et al. 1977a,b) (see Table 1.1) are maintained in each bacterial cell (Covarrubias et al. 1981). Such multicopy plasmids are said to replicate in a "relaxed" fashion. The pMB1 (or ColE1) replicon does not require plasmid-encoded functions for replication (Tomizawa et al. 1974); instead, it relies entirely on long-lived enzymes supplied by the host (DNA polymerases I and III, DNA-dependent RNA polymerase, and the products of the host genes *dnaB, dnaC, dnaD,* and *dnaZ*) (for review, see Scott 1984). It can therefore function in the absence of ongoing protein synthesis (for review, see Staudenbauer 1978). Thus, in the presence of antibiotics such as chloramphenicol or spectinomycin, which inhibit protein synthesis and prevent replication of the bacterial chromosome, plasmids carrying the pMB1 (or ColE1) replicon will continue to replicate until two or three thousand copies have accumulated in the cell (Clewell 1972).

Replication occurs unidirectionally from a specific origin and is primed by an RNA primer whose promoter lies about 550 bases upstream of the origin of replication (Figure 1.1). Persistent hybrids formed between the template strand of DNA and the nascent RNA serve as substrates for the enzyme RNAase H, which cleaves the preprimer to generate the primer for DNA synthesis (known as RNA II) (Itoh and Tomizawa 1980). The maturation of RNA II is controlled by another small, untranslated RNA molecule (RNA I), which is transcribed from the opposite strand of the same region of DNA that codes for RNA II. RNA I binds to RNA II and prevents its folding into a cloverleaf structure that is necessary for the formation of stable DNA:RNA hybrids between RNA II and the plasmid DNA (see Tomizawa 1984; Dooley and Polisky 1987). This binding between RNAs I and II is enhanced by a small protein of 63 amino acids (the Rop protein), which is encoded by a gene located 400 nucleotides downstream from the origin of replication (Cesareni et al. 1982; Tomizawa and Som 1984). Consequently, the Rop protein

reinforces the negative regulation of replication by RNA I (for review, see Cesareni and Banner 1985).

The copy number of plasmids carrying the pMB1 (or ColE1) replicon can therefore be increased by mutations that weaken the interaction between RNAs I and II. These mutations map within the *rop* gene or within the region upstream of the origin that codes for RNAs I and II (for review, see Scott 1984). For example, pUC plasmids have a high copy number because they carry a mutation (G→A) one nucleotide upstream of the normal site of initiation of RNA I (Minton et al. 1988). This results in RNA I transcripts that are initiated three nucleotides further downstream than usual. The integrity of the 5′ single-stranded domain of RNA I is crucial for the interaction of RNA I with RNA II (Dooley and Polisky 1987). It therefore seems likely that the increased copy number of pUC plasmids is caused by inefficient binding of truncated RNA I to RNA II.

In addition to controlling copy number, the regions of the plasmid encoding RNA I, RNA II, and the Rop protein also determine whether two different plasmids will coexist in the same bacterial cell (for review, see Davison 1984). Plasmids that utilize the same replication system cannot coexist stably and are said to be incompatible (Datta 1979). When two such plasmids are introduced into the same cell, they compete with one another both during replication and during the subsequent step of partition into daughter cells. Because plasmid molecules are selected at random from the intracellular pool for replication, the copy numbers of two different plasmids in an individual cell will not always remain equal. Small differences, generated originally by stochastic processes, lead rapidly to more severe imbalances in the copy numbers of the two plasmids. In some cells, one plasmid dominates; in others, its incompatible partner prospers. Over the course of a few generations of bacterial growth, the minority plasmid is completely eliminated and the descendants of the original cell contain one plasmid or the other, but not both. By testing the ability of different plasmids to coexist in the same cell, it is possible to assign them to incompatibility groups. Plasmids carrying the same replicon belong to the same incompatibility group; plasmids carrying replicons whose components are not interchangeable belong to different groups. Over 30 such groups have now been recognized (Datta 1979).

The replicons carried by plasmid vectors in current use are shown in Table 1.1. Plasmids carrying the ColE1 and pMB1 replicons are incompatible with one another, but they are fully compatible with those carrying pSC101 and p15A replicons (Chang and Cohen 1978; Selzer et al. 1983). The pSC101 replicon, which has not been analyzed in great detail, may resemble the replicon of plasmid R6-5, from which it is partially derived (Cohen and Chang

TABLE 1.1 Replicons Carried by Currently Used Plasmid Vectors

Plasmid	Replicon	Copy number
pBR322 and its derivatives	pMB1	15–20
pUC vectors	pMB1	500–700
pACYC and its derivatives	p15A	10–12
pSC101 and its derivatives	pSC101	~5
ColE1	ColE1	15–20

1977). The R6-5 replicon differs from the others shown in Table 1.1 in that it codes for an essential *cis*-acting protein (Linder et al. 1985; Womble et al. 1985). This protein acts positively on the origin of replication and regulates negatively the transcription of its own gene (the *repA* gene). Plasmids like pSC101 are therefore under stringent replicative control and are present at only 5 copies per cell or less. Because stringently controlled replication requires ongoing expression of a plasmid-encoded protein, the copy number of these plasmids cannot be increased by inhibitors of protein synthesis such as chloramphenicol. Most cloning tasks are therefore carried out in relaxed vectors because they give a greater yield of DNA per unit volume of culture and, in many cases, an improved yield of protein products synthesized from cloned genes. However, under some circumstances, the presence of many copies of the cloned gene and/or its product may be deleterious. The use of stringently controlled plasmids may then permit the isolation of functional genes that are lethal when expressed in high amounts from a multicopy plasmid. Examples of such genes include *polA* (Murray and Kelley 1979), *dnaA* (Hansen and von Meyenburg 1979), *ompA* (Beck and Bremer 1980), and *galK* (Davison et al. 1984). For descriptions of low-copy-number plasmid vectors, see Kahn et al. (1979) and Stoker et al. (1982).

Mobilization

Under natural conditions, many plasmids are transmitted to new hosts by a process known as bacterial conjugation (for review, see Willetts and Wilkins 1984). However, plasmid vectors in common use lack a gene (*mob*) that is required for mobilization and are incapable of directing their own conjugal transfer from one bacterium to another. Nevertheless, some of the older plasmid vectors (e.g., pBR322) can be mobilized by a conjugative plasmid if a third plasmid (ColK) is present in the cell (Young and Poulis 1978). ColK is thought to code for a mobility protein that nicks pBR322 at a site (*nic*) close to the *cis*-acting element *bom*. Mobilization of the plasmid then occurs from this nicked site (nucleotide 2254 in the pBR322 sequence). Newer plasmid vectors (e.g., the pUC vectors) lack the *nic*/*bom* site and therefore cannot be mobilized (Twigg and Sherratt 1980; Covarrubias et al. 1981).

Selectable Markers

In the laboratory, plasmid DNA can be introduced into bacteria by the artificial process of transformation. In this process, bacteria are treated with mixtures of divalent cations to make them temporarily permeable to small DNA molecules. Even under the best conditions, plasmids become stably established in only a small minority of the bacterial population. To identify these transformants, selectable markers encoded by the plasmid are used. These markers confer a new phenotype, which allows bacteria that have been successfully transformed to be selected with ease.

The most commonly used selectable markers are genes that confer resistance to antibiotics such as ampicillin, tetracycline, chloramphenicol, and kanamycin (neomycin). Each of these antibiotics operates through a different mechanism (for review, see Davies and Smith 1978):

- *Tetracycline* binds to a protein of the 30S subunit of the ribosome and inhibits ribosomal translocation. The constitutively expressed tetracycline resistance (*tet*^{r}) gene carried on plasmid pBR322 encodes a 399-amino-acid, membrane-associated protein (Backman and Boyer 1983) that prevents the antibiotic from entering the cell (Franklin 1967).
- *Ampicillin* binds to and inhibits a number of enzymes in the bacterial membrane that are involved in the synthesis of the cell wall (for review, see Waxman and Strominger 1983). The ampicillin resistance (*amp*^{r}) gene carried on the plasmid codes for an enzyme that is secreted into the periplasmic space of the bacterium, where it catalyzes hydrolysis of the β-lactam ring, with concomitant detoxification of the drug (Sykes and Mathew 1976).
- *Chloramphenicol* binds to the ribosomal 50S subunit and inhibits protein synthesis. The chloramphenicol resistance (*Cm*^{r} or *cat*) gene used in current plasmid vectors was originally isolated from a transducing P1 bacteriophage (Kondo and Mitsuhashi 1964) that was later shown to carry the transposon Tn*9*. The *cat* gene codes for a tetrameric, cytosolic protein (M_r of each subunit = 23,000) that, in the presence of acetyl coenzyme A, catalyzes the formation of hydroxyl acetoxy derivatives of chloramphenicol that are unable to bind to ribosomes (Shaw et al. 1979). The expression of chloramphenicol acetyltransferase is catabolite-sensitive and is increased five- to tenfold when bacteria are grown on carbon sources other than glucose. Binding of DNA-dependent RNA polymerase to the promoter of the *cat* gene in vitro is increased markedly by the presence of the cAMP/catabolite gene activator protein (Le Grice and Matzura 1981).
- *Kanamycin and neomycin* are deoxystreptamine aminoglycosides that bind to ribosomal components and inhibit protein synthesis. Both antibiotics are inactivated by an aminoglycoside phosphotransferase (APH[3′]-II) with a molecular weight of 25,000 that appears to be located in the periplasmic space. Phosphorylation of these antibiotics is believed to interfere with their active transport into the cell (for review, see Davies and Smith 1978).

Virtually all plasmid vectors in common use carry one or more of the antibiotic resistance genes described above. However, other selectable markers are occasionally used for more specialized purposes. Perhaps the most elegant of these is *supF*, the gene that codes for the bacterial suppressor tRNA (Seed 1983). This small (203-bp) gene is used in the plasmid πVX (Seed 1983; see Maniatis et al. 1982 for map) and its updated version πAN13 (Lutz et al. 1987) to suppress amber mutations in *tet*^{r} and *amp*^{r} genes carried on a second, compatible plasmid. This suppression confers ampicillin and tetracycline resistance upon cells containing both plasmids.

U.S. Pat. No. 5,695,976, "Stable Integration of DNA in Bacterial Genomes," issued December 9, 1997

US005695976A

United States Patent [19]

Jørgensen et al.

[11] Patent Number: 5,695,976

[45] Date of Patent: Dec. 9, 1997

[54] **STABLE INTEGRATION OF DNA IN BACTERIAL GENOMES**

[75] Inventors: **Steen Troels Jørgensen**, Allerød; **Per Lind Jørgensen**, Copenhagen; **Børge Krag Diderichsen**, Birkerød, all of Denmark

[73] Assignee: **Novo Nordisk A/S**, Bagsvaerd, Denmark

[21] Appl. No.: **441,714**

[22] Filed: **May 15, 1995**

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[30] **Foreign Application Priority Data**

Dec. 18, 1989 [DK] Denmark 6396/89

[51] **Int. Cl.**[6] **C12N 15/64**; C12N 15/65; C12N 15/75; C12P 21/02

[52] **U.S. Cl.** **435/172.3**; 435/69.1; 435/320.1

[58] **Field of Search** 435/69.1, 252.35, 435/172.3, 320.1, 252.31

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Primary Examiner—Nancy Degen
Attorney, Agent, or Firm—Steve T. Zelson; Cheryl H. Agris

[57] **ABSTRACT**

A bacterial cell which in its genome carries an integrated non-replicative DNA construct comprising (1) a DNA sequence of interest, (2) a DNA sequence which is homologous with a region of the genome of the cell, and (3) an origin of replication, the DNA construct lacking a functional gene coding for a factor required to initiate replication from the origin of replication.

49 Claims, 33 Drawing Sheets

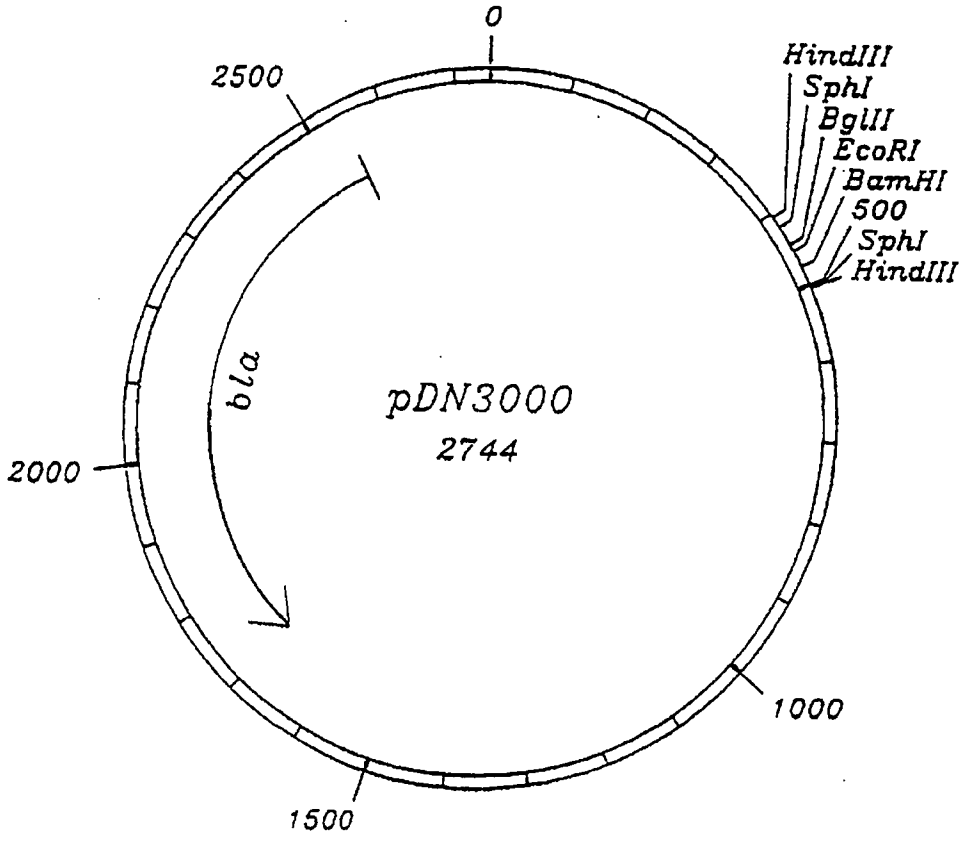
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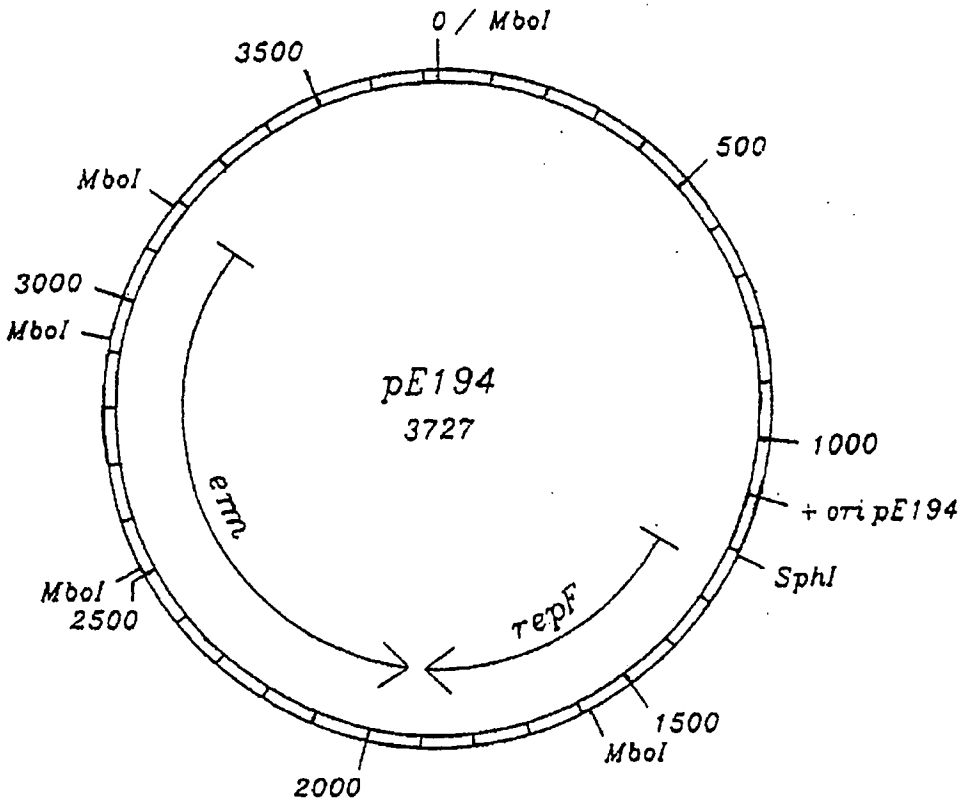
FIG. 1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repF

FIG. 2

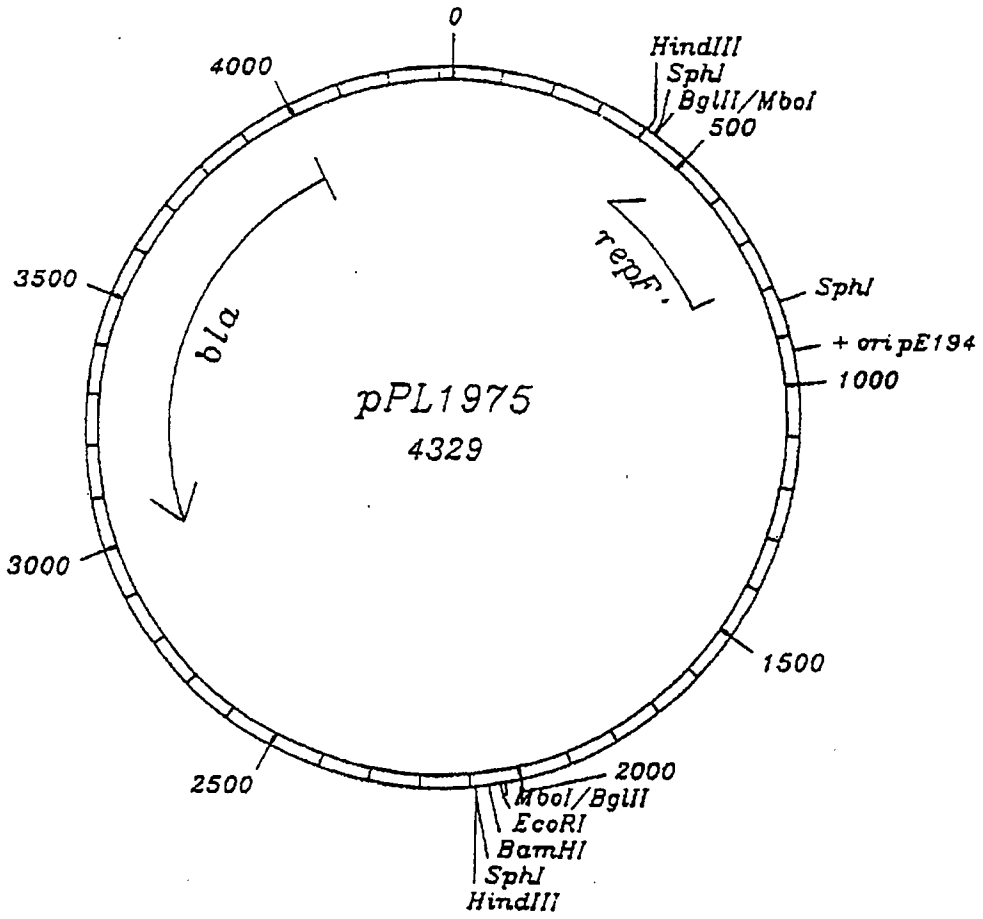

0
HindIII
SphI
BglII/MboI
500
1000
+ oriE194
SphI
1500
2000
MboI/BglII
EcoRI
BamHI
SphI
HindIII
2500
3000
3500
4000
bla
repF
pPL1975
4329

FIG. 3

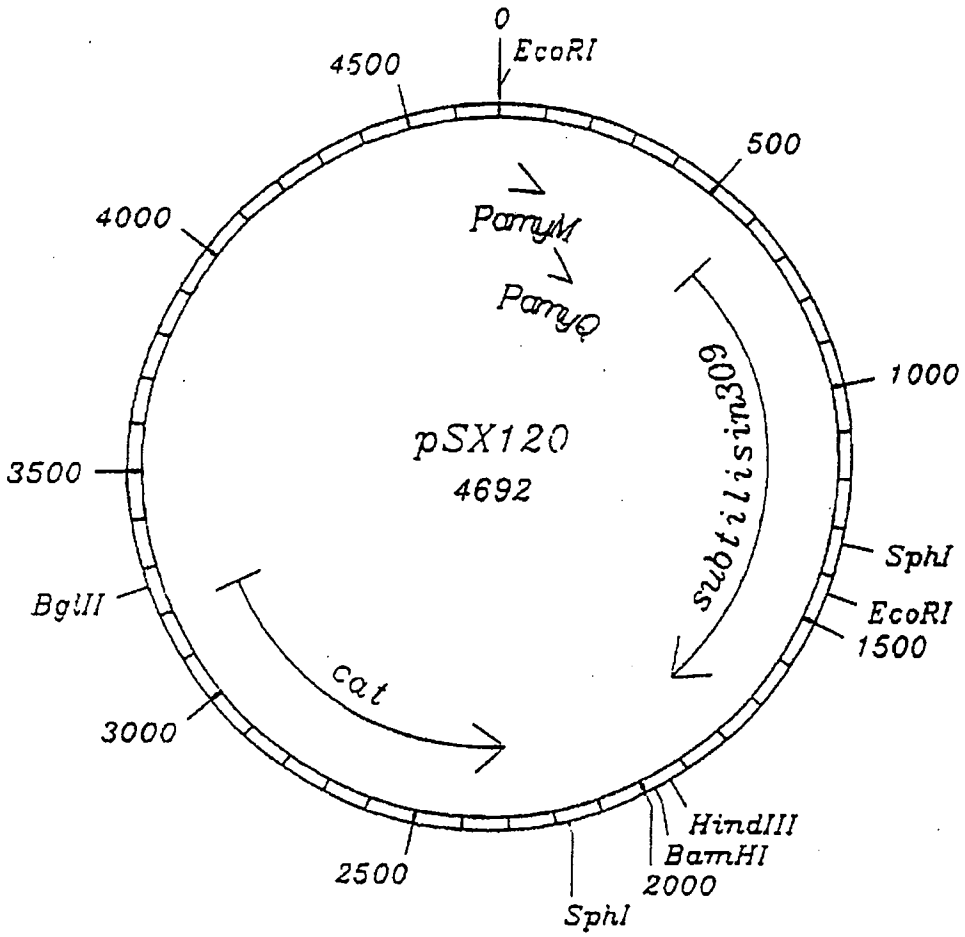

0
EcoRI
500
1000
SphI
EcoRI
1500
HindIII
BamHI
2000
SphI
2500
3000
BglII
3500
4000
4500
ParyM
ParyQ
pSX120
4692
subtilisin309
cat

FIG. 4

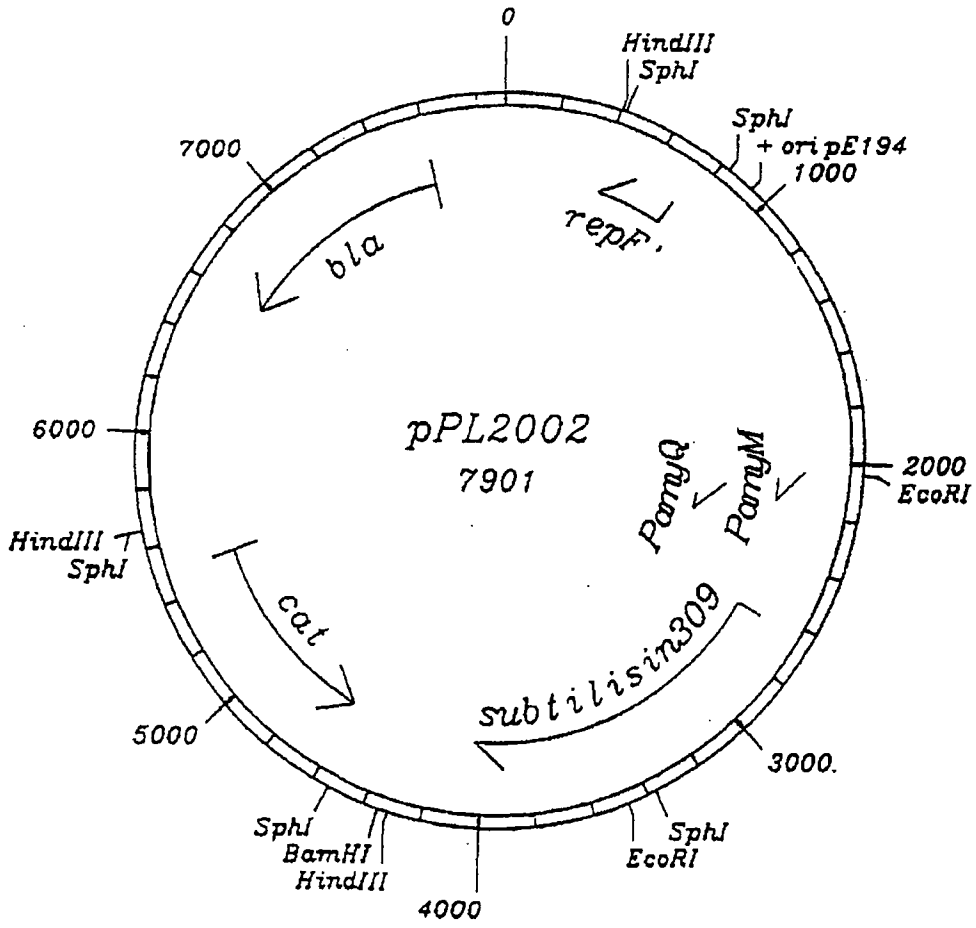

0
HindIII
SphI
SphI
+ ori pE194
1000
2000
EcoRI
3000.
SphI
EcoRI
4000
SphI
BamHI
HindIII
5000
HindIII
SphI
6000
7000
bla
repF
pPL2002
7901
PamyQ
PamyM
cat
subtilisin309

FIG. 5

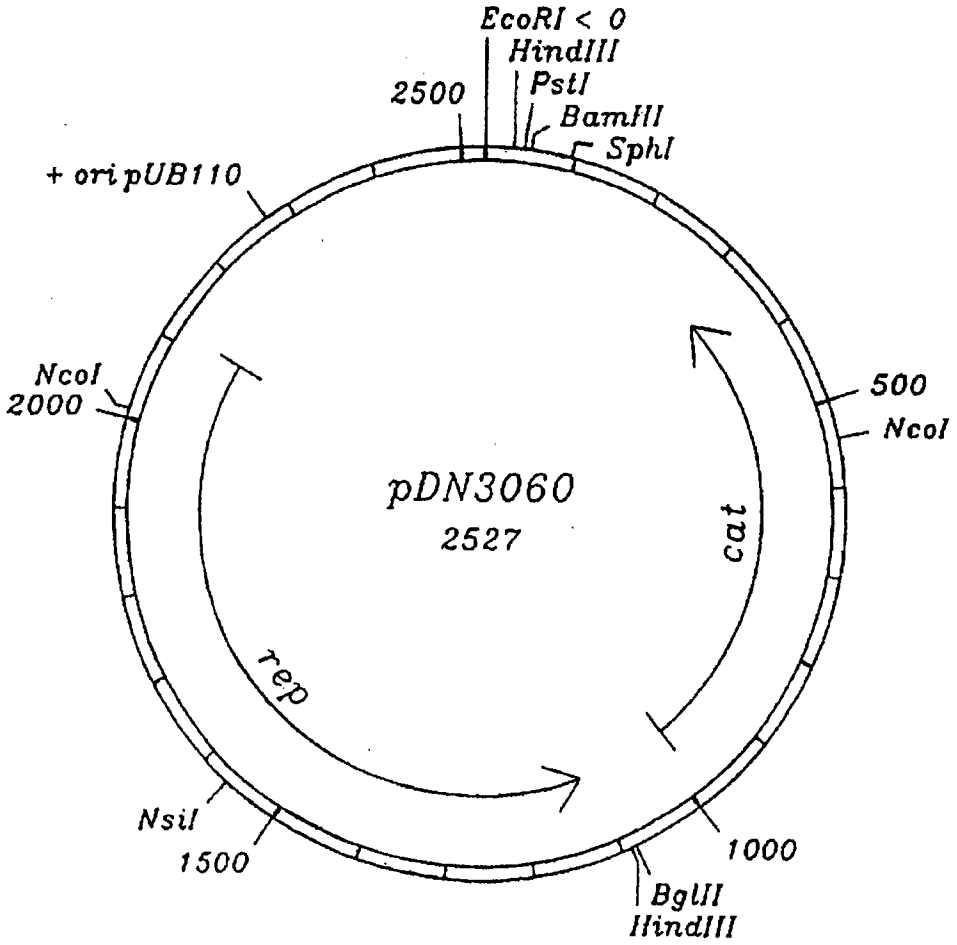

EcoRI < 0
HindIII
PstI
BamHI
SphI
2500
+ ori pUB110
NcoI
2000
500
NcoI
pDN3060
2527
cat
rep
NsiI
1500
1000
BglII
HindIII

FIG. 6

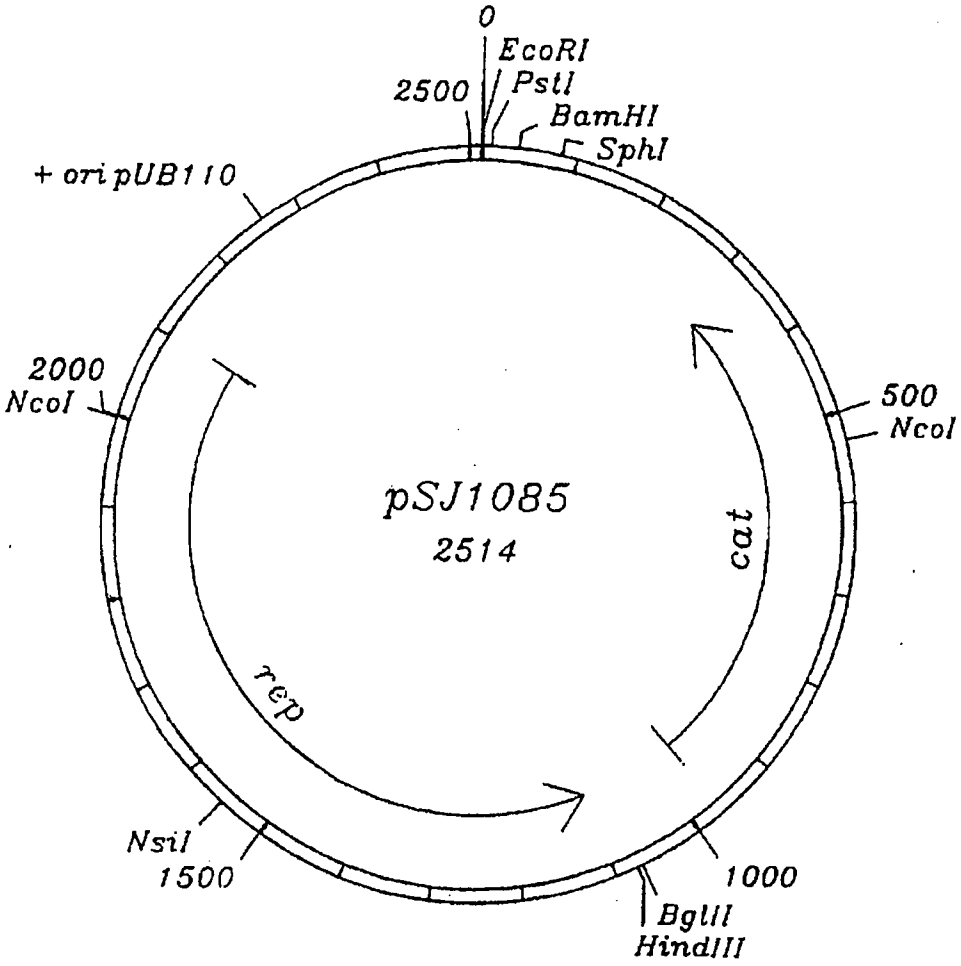

0
EcoRI
PstI
2500
BamHI
SphI
+ ori pUB110
2000
NcoI
500
NcoI
pSJ1085
2514
cat
rep
NsiI
1500
1000
BglII
HindIII

FIG. 7

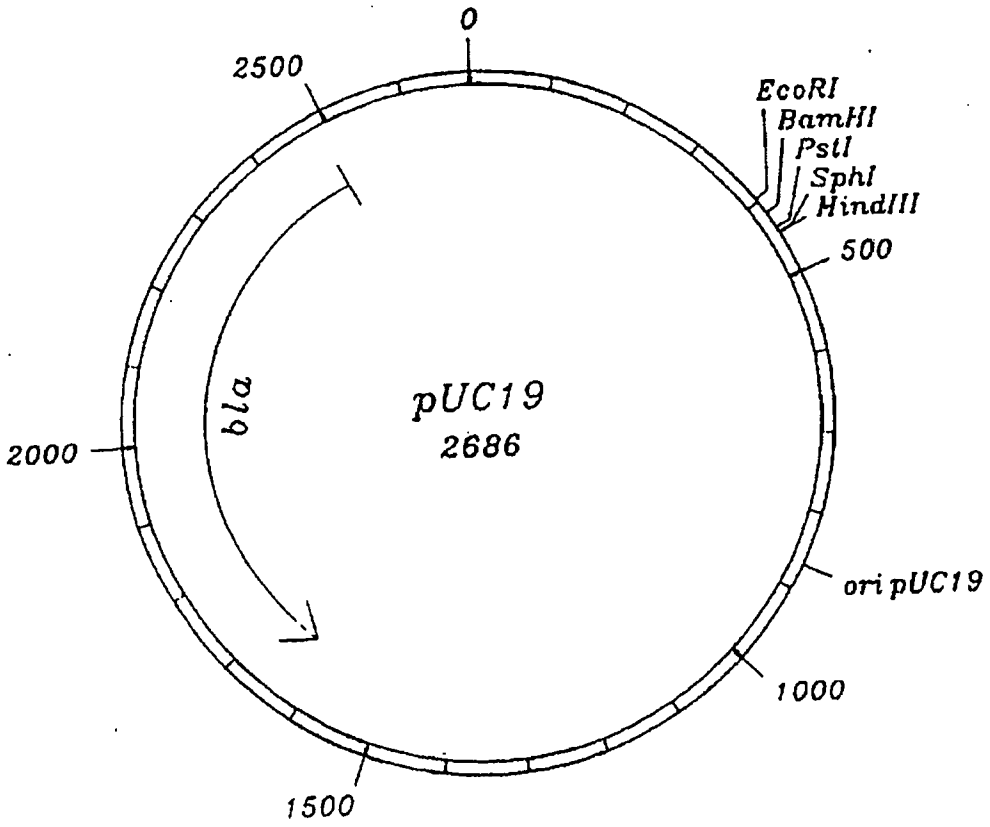

0
2500
EcoRI
BamHI
PstI
SphI
HindIII
500
bla
pUC19
2686
2000
ori pUC19
1000
1500

FIG. 8

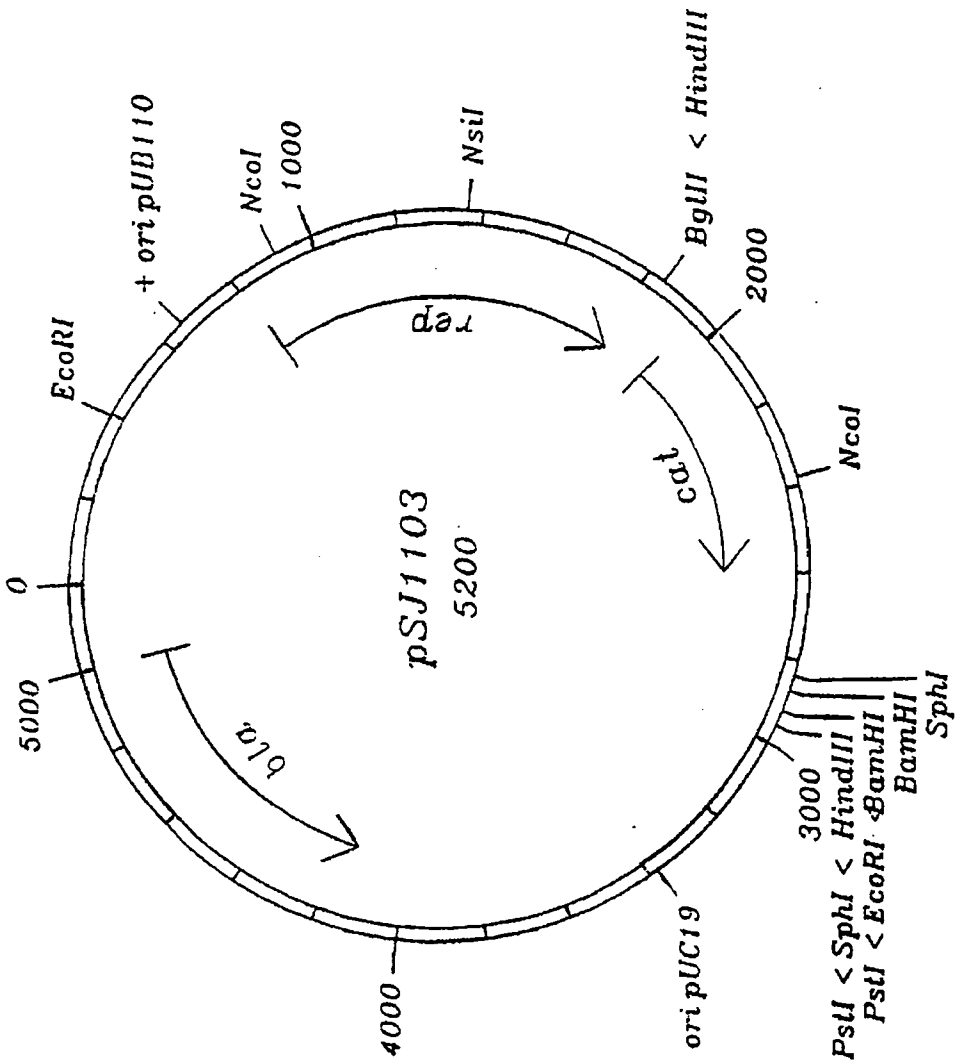

pSJ1103
5200
rep
cat
blo
0
1000
2000
3000
4000
5000
+ ori pUB110
NcoI
NsiI
BglII < HindIII
NcoI
EcoRI
ori pUC19
PstI < SphI < HindIII
PstI < EcoRI < BamHI
BamHI
SphI

FIG. 9

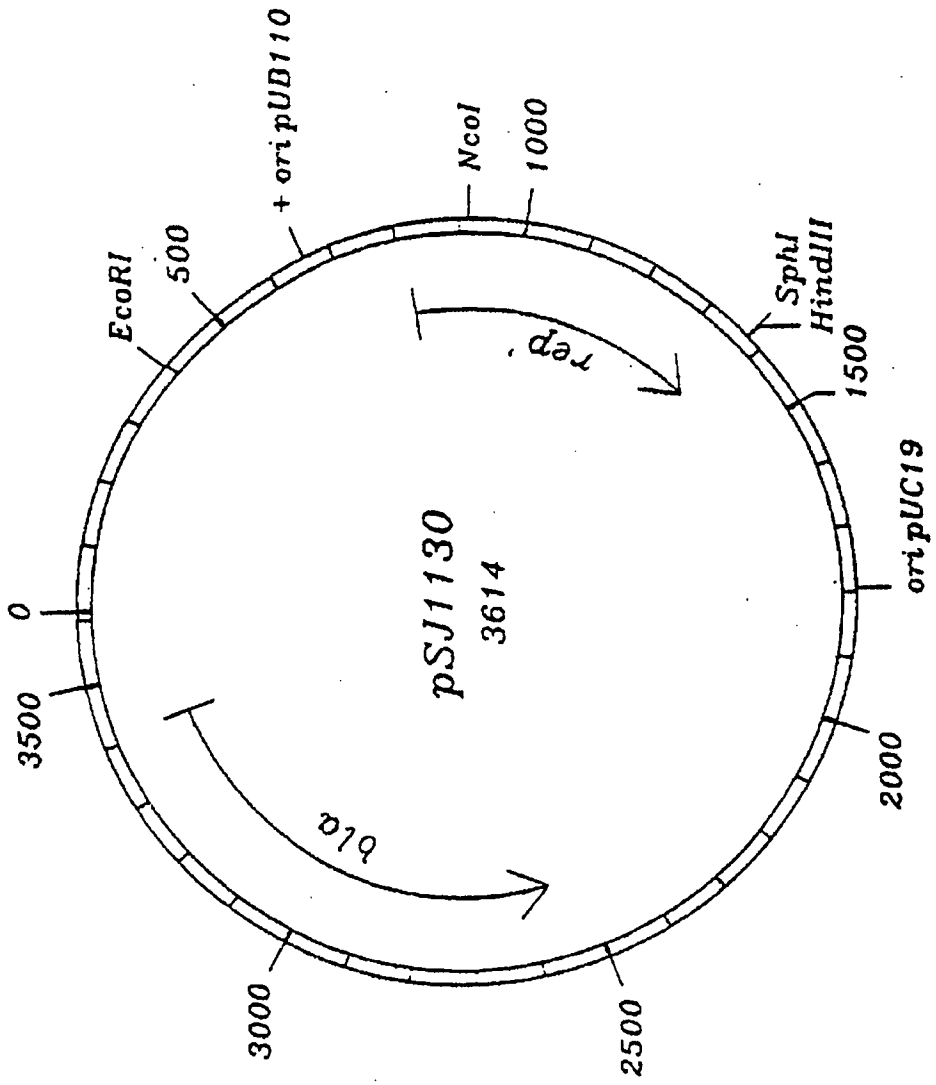

+ ori pUB110
NcoI
1000
SphI
HindIII
1500
ori pUC19
2000
2500
3000
3500
0
EcoRI
500
Rep
bla
pSJ1130
3614

FIG. 10

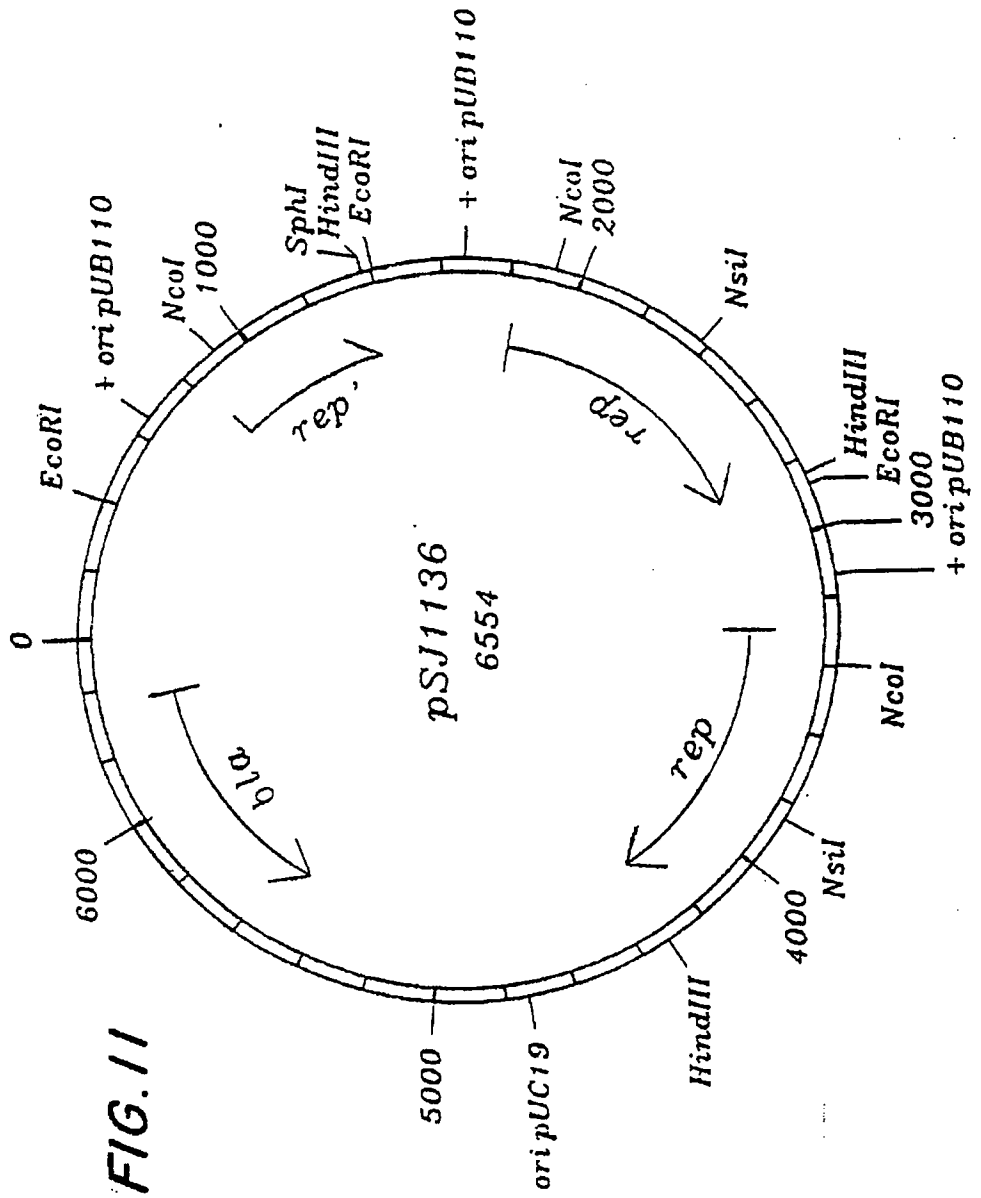

pSJ1136
6554
0
EcoRI
+ ori pUB110
NcoI
1000
SphI
HindIII
EcoRI
+ ori pUB110
NcoI
2000
NsiI
HindIII
EcoRI
3000
+ ori pUB110
NcoI
NsiI
4000
HindIII
5000
ori pUC19
6000
rep
rep
rep
rep

FIG. 11

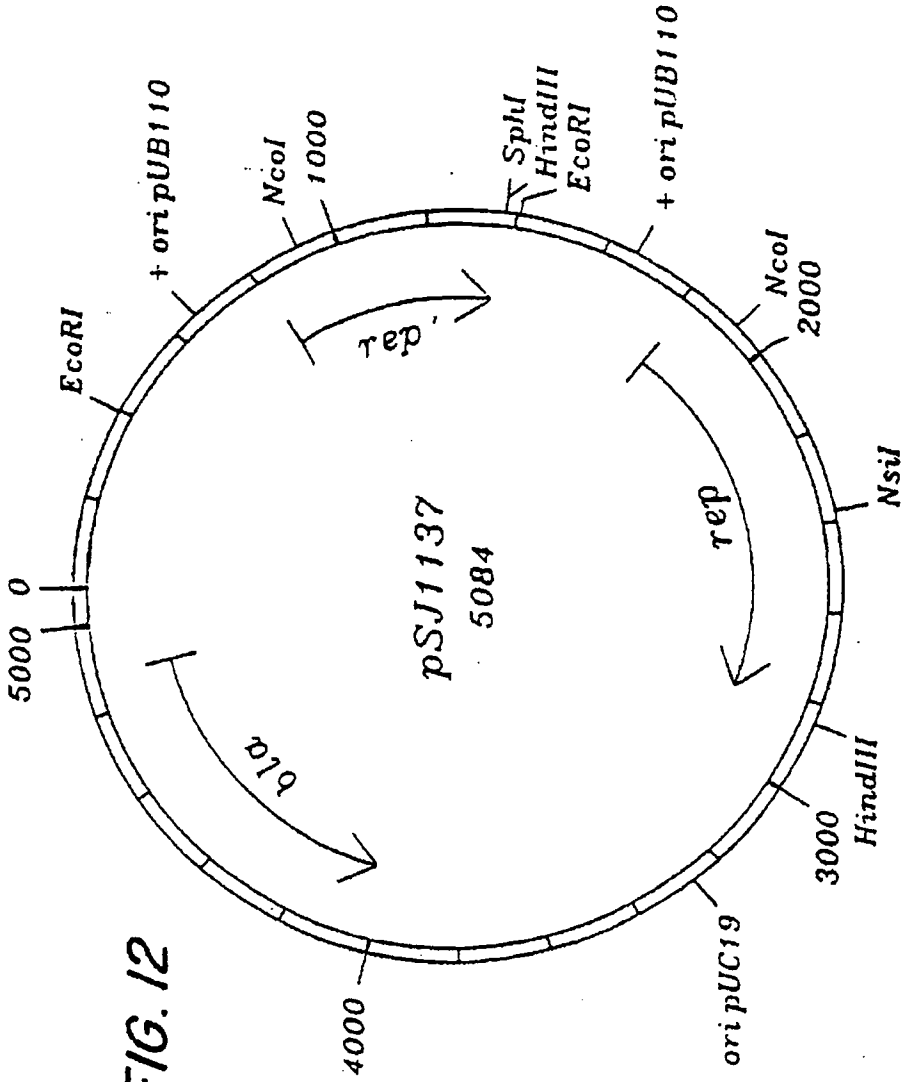

pSJ1137
5084
rep
rep
rop
0
1000
2000
3000
4000
5000
EcoRI
+ ori pUB110
NcoI
SphI
HindIII
EcoRI
+ ori pUB110
NcoI
NsiI
HindIII
ori pUC19

FIG. 12

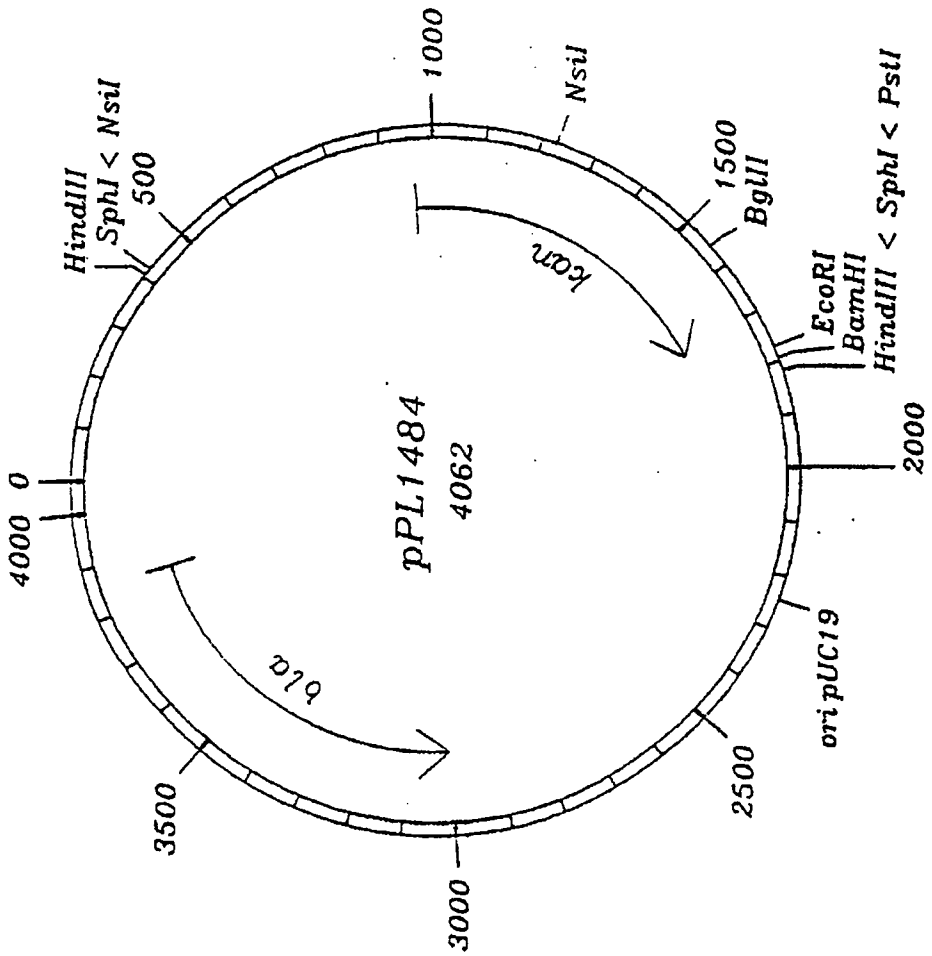

1000
NsiI
1500
BglII
EcoRI
BamHI
HindIII < SphI < PstI
2000
ori pUC19
2500
3000
3500
4000 0
HindIII
SphI < NsiI
500
kan
pPL1484
4062
tet

FIG.13

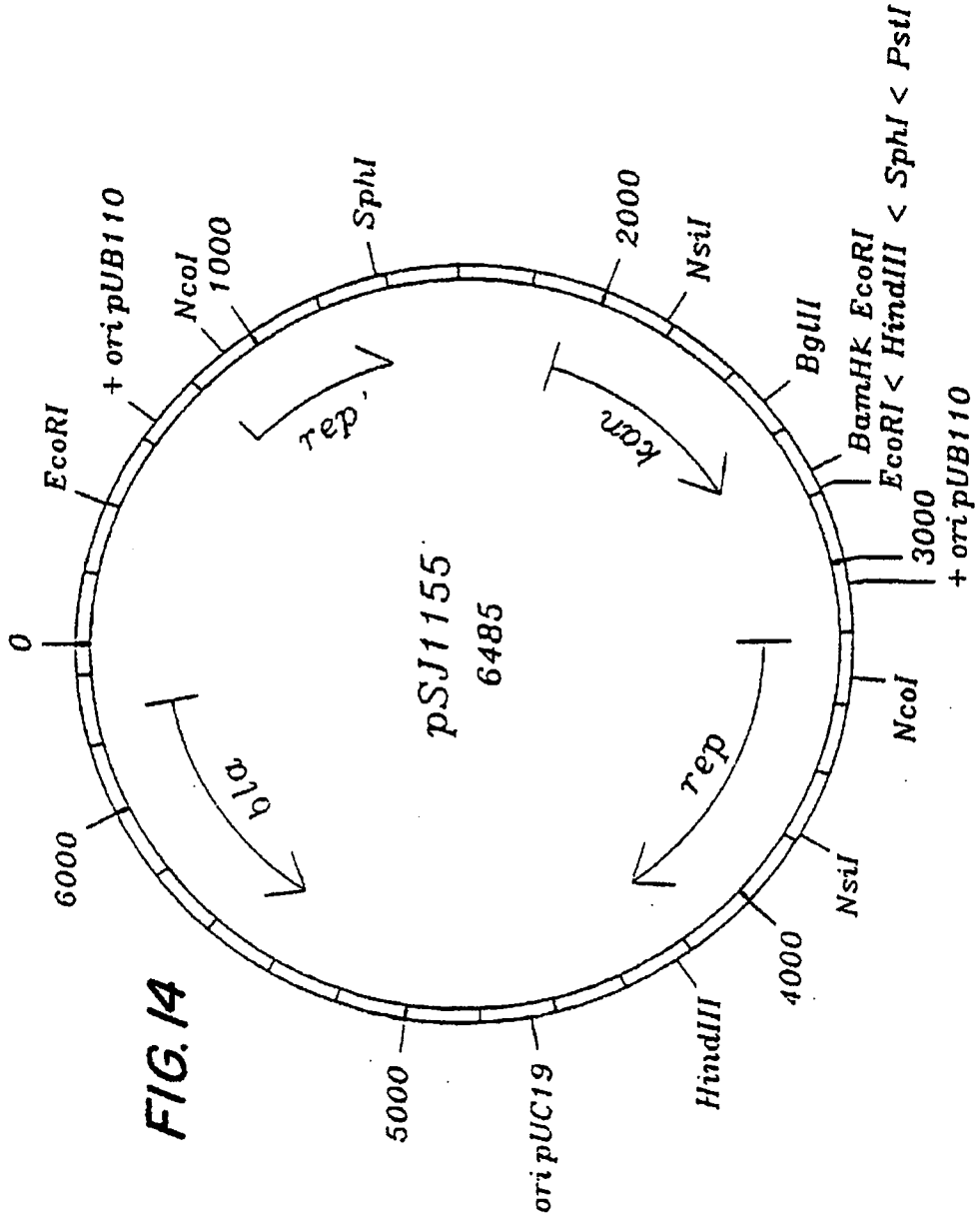

pSJ1155
6485
0
1000
2000
3000
4000
5000
6000
EcoRI
+ ori pUB110
NcoI
SphI
NsiI
BglII
BamHK EcoRI
EcoRI < HindIII < SphI < PstI
+ ori pUB110
NcoI
NsiI
HindIII
ori pUC19
rep
ori
rep
kan

FIG. 14

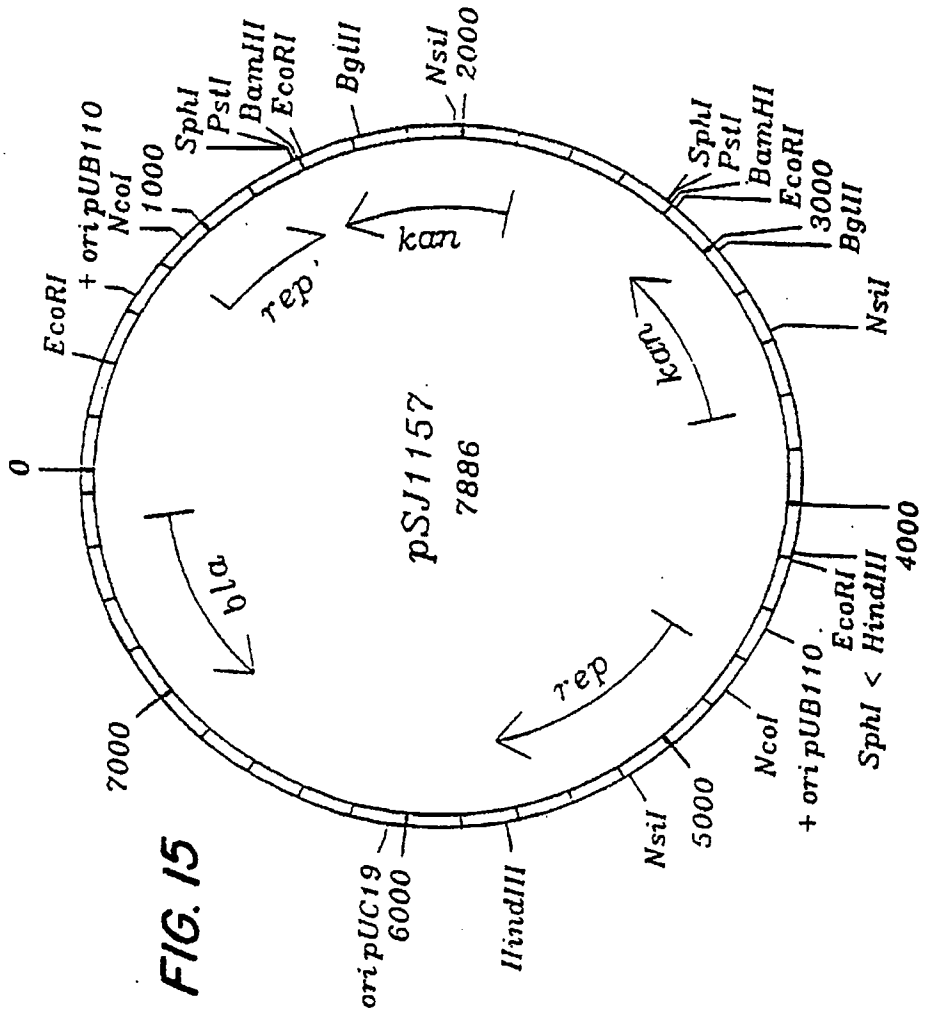

pSJ1157
7886
0
1000
2000
3000
4000
5000
6000
7000
kan
rep
bla
rep
kan
ori pUC19
HindIII
NsiI
NcoI
+ ori pUB110
EcoRI
SphI < HindIII
EcoRI
BamHI
PstI
SphI
BglII
NsiI
EcoRI
BamHI
PstI
SphI
BglII
NsiI
NcoI
+ ori pUB110
EcoRI

FIG. 15

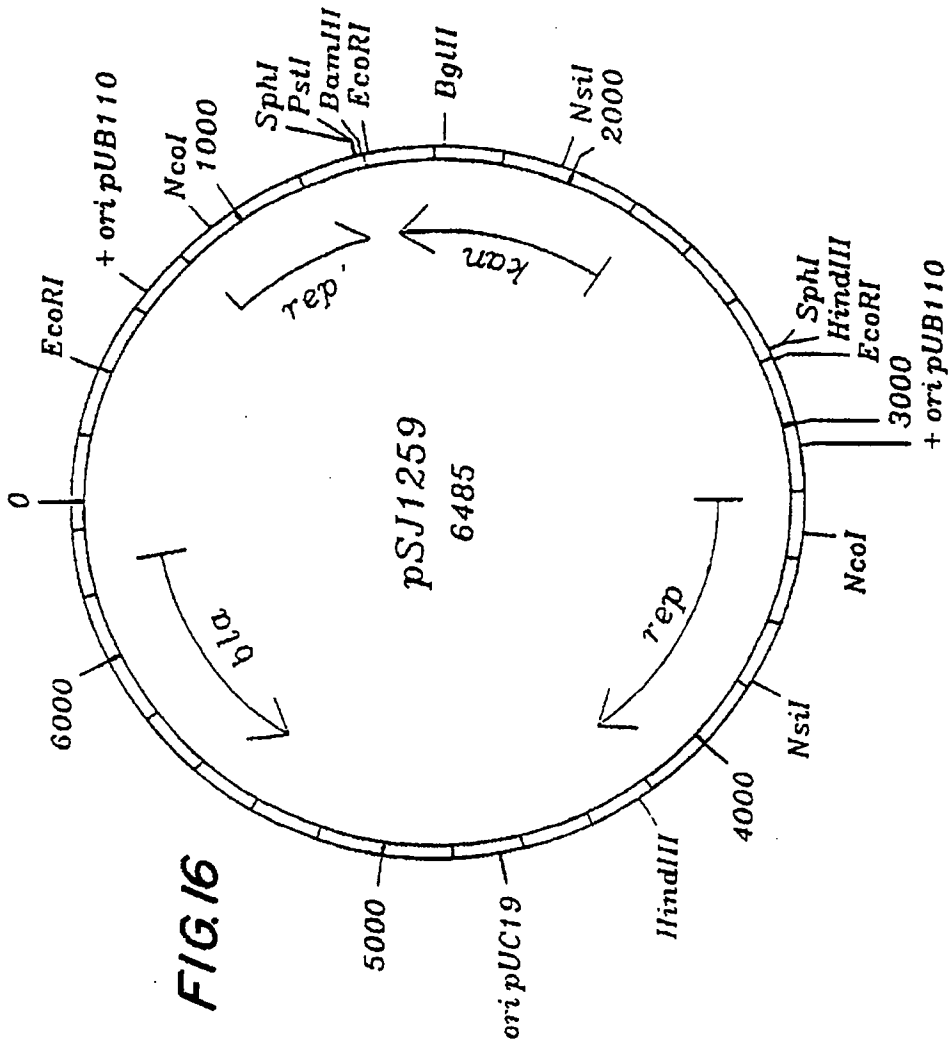

pSJ1259
6485
0
1000
2000
3000
4000
5000
6000
EcoRI
+ ori pUB110
NcoI
SphI
PstI
BamHI
EcoRI
BglII
NsiI
SphI
HindIII
EcoRI
+ ori pUB110
NcoI
NsiI
HindIII
ori pUC19
rep
bla
rep
kan

FIG. 16

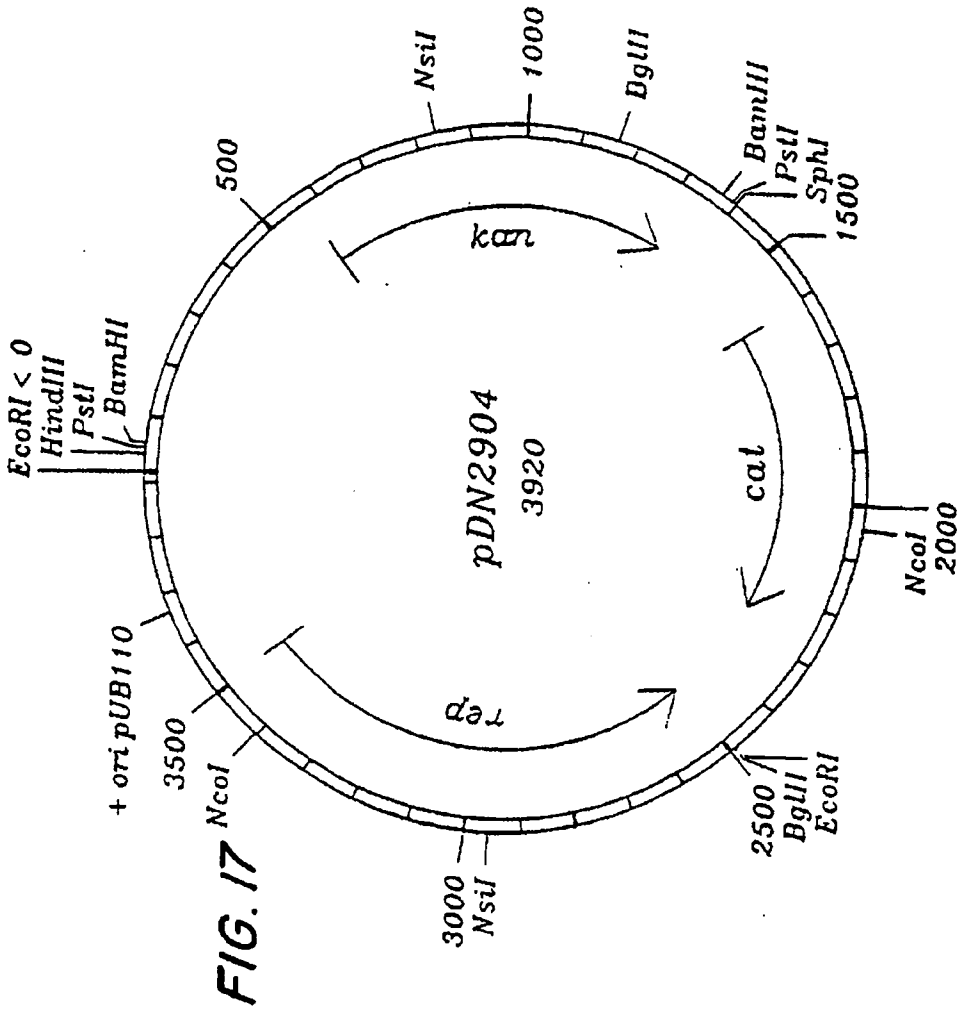

pDN2904
3920
kan
cat
rep
EcoRI < 0
HindIII
PstI
BamHI
500
NsiI
1000
BglII
BamHI
PstI
SphI
1500
NcoI
2000
2500
BglII
EcoRI
3000
NsiI
NcoI
3500
+ ori pUB110

FIG. 17

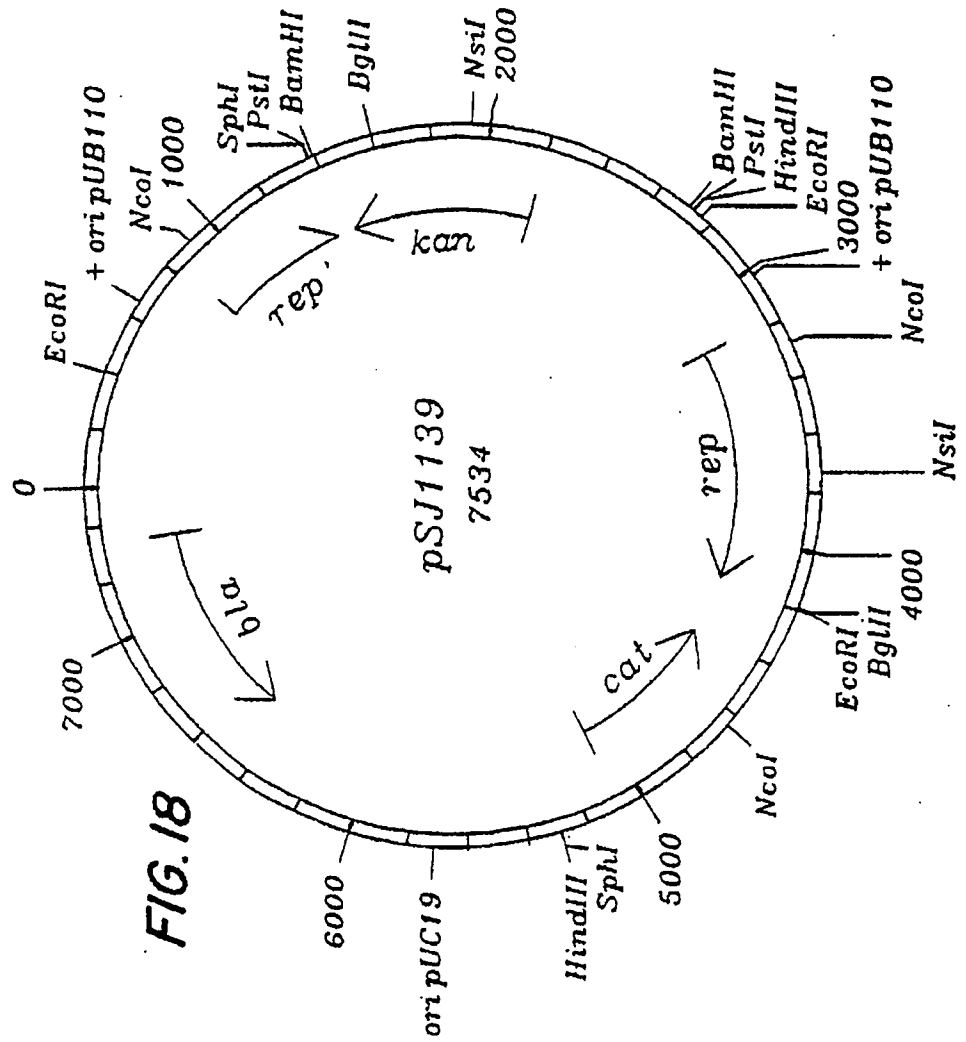

pSJ1139
7534
kan
rep.
rep
bla
cat
0
1000
2000
3000
4000
5000
6000
7000
EcoRI
+ ori pUB110
NcoI
SphI
PstI
BamHI
BglII
NsiI
BamHI
PstI
HindIII
EcoRI
+ ori pUB110
NcoI
NsiI
EcoRI
BglII
NcoI
HindIII
SphI
ori pUC19

FIG. 18

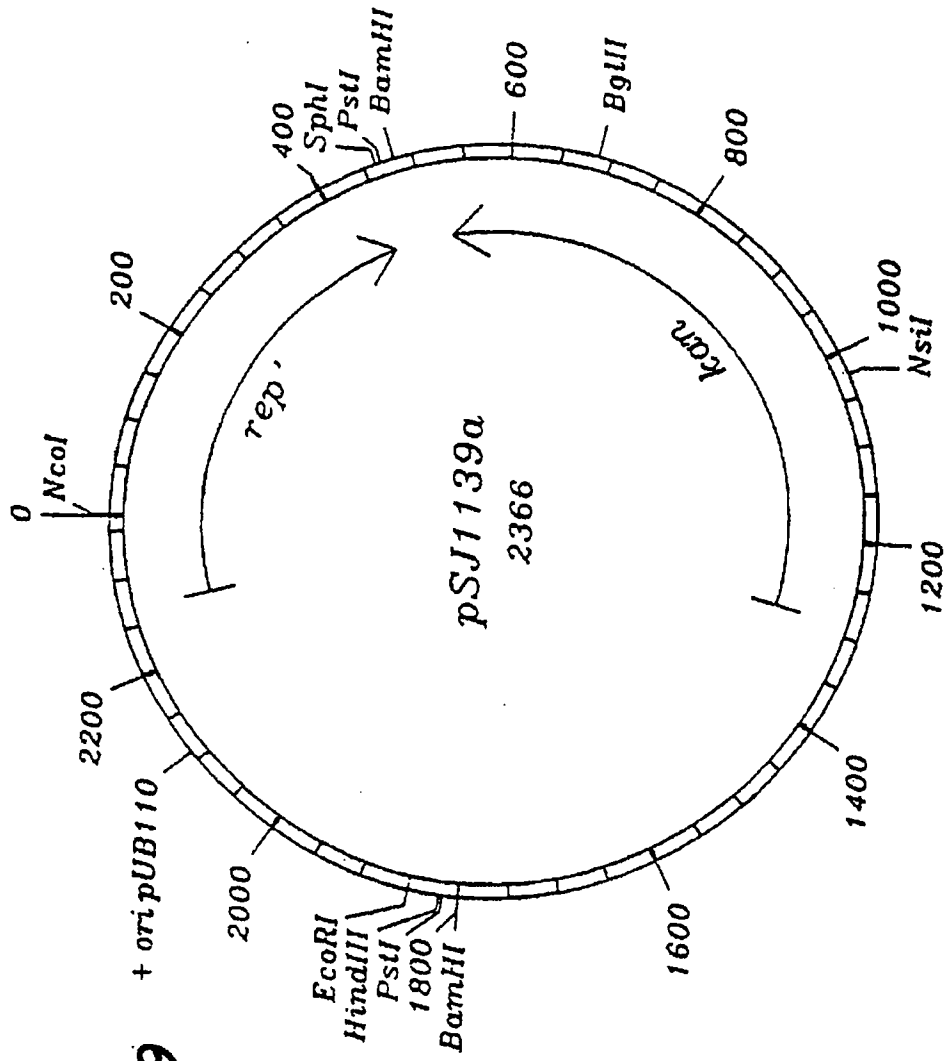

0
NcoI
200
400
SphI
PstI
BamHI
600
BglII
800
1000
NsiI
1200
1400
1600
1800
BamHI
PstI
HindIII
EcoRI
2000
2200
+ ori pUB110
rep.
cm
pSJ1139a
2366

FIG. 19

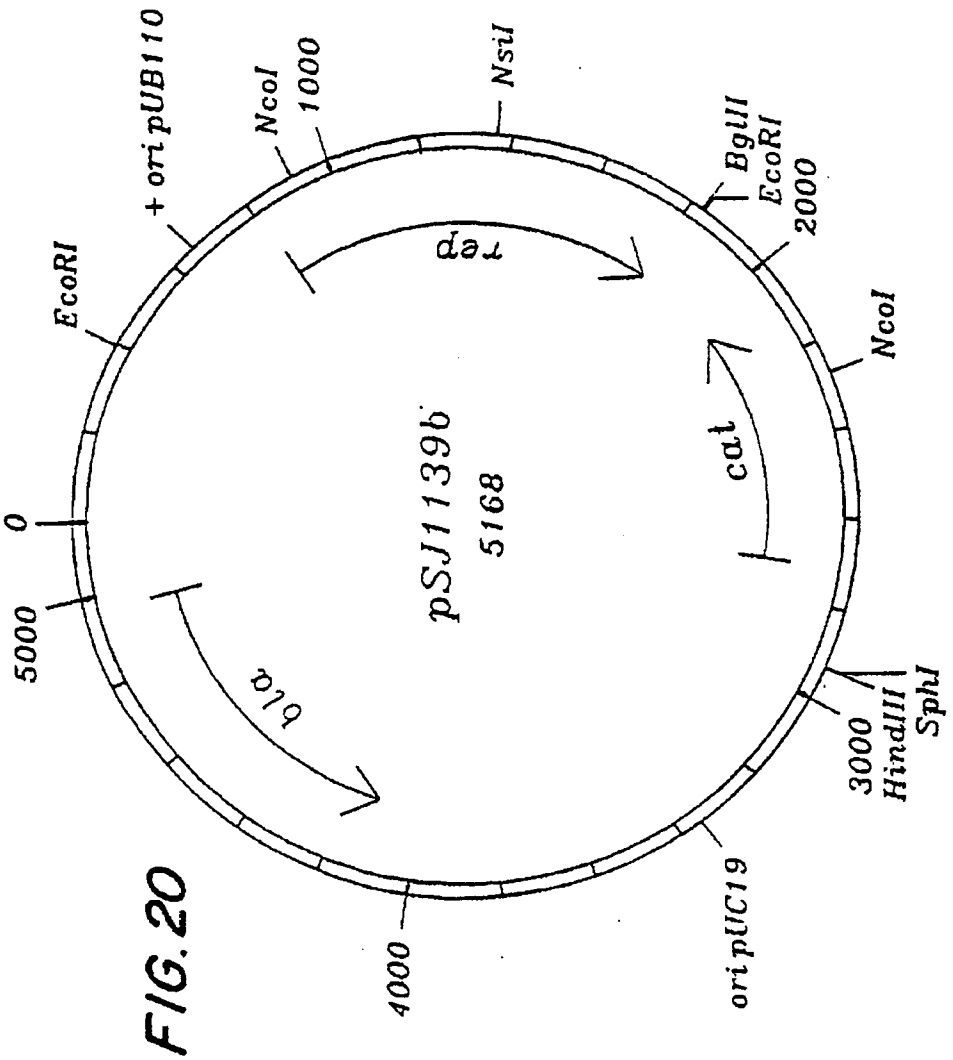

pSJ11396
5168
rep
cat
bla
0
1000
2000
3000
4000
5000
EcoRI
+ oriPUB110
NcoI
NsiI
BglII
EcoRI
NcoI
HindIII
SphI
oriPUC19

FIG. 20

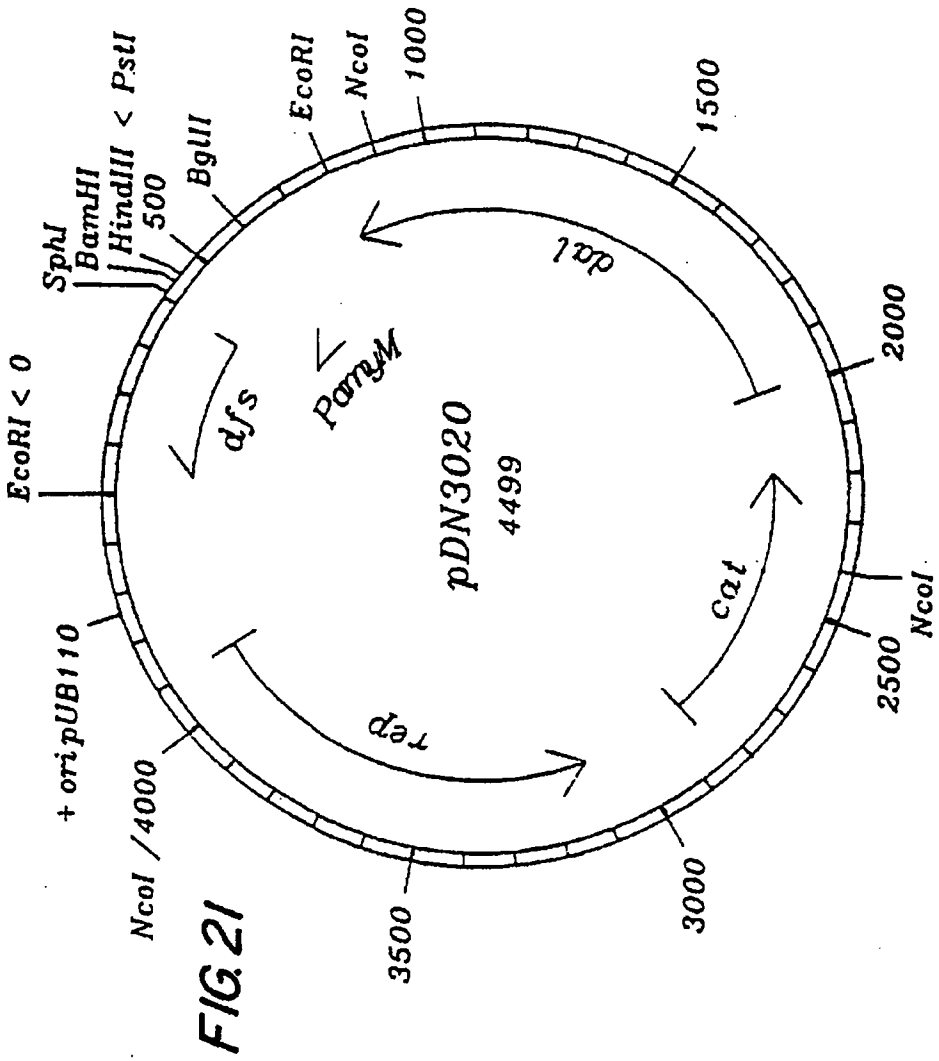

pDN3020
4499
dfs
ParaM
top
cat
Tep
EcoRI < 0
SphI
BamHI
HindIII < PstI
500
BglII
EcoRI
NcoI
1000
1500
2000
NcoI
2500
3000
3500
NcoI /4000
+ oripUB110

FIG 21

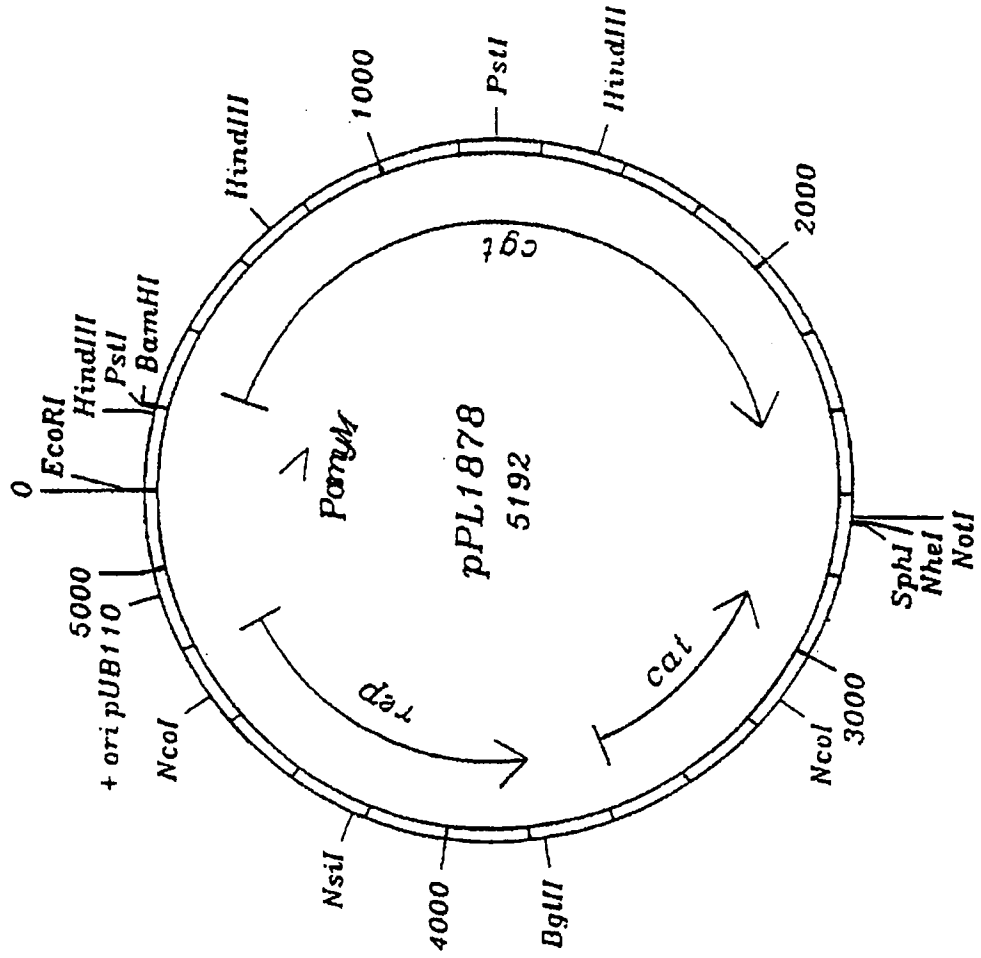

0
EcoRI
HindIII
PstI
BamHI
HindIII
1000
PstI
HindIII
2000
SphI
NheI
NotI
3000
NcoI
cat
4000
BglII
NsiI
repB
NcoI
+ ori pUB110
5000
Porgur
pPL1878
5192
rep

FIG. 22

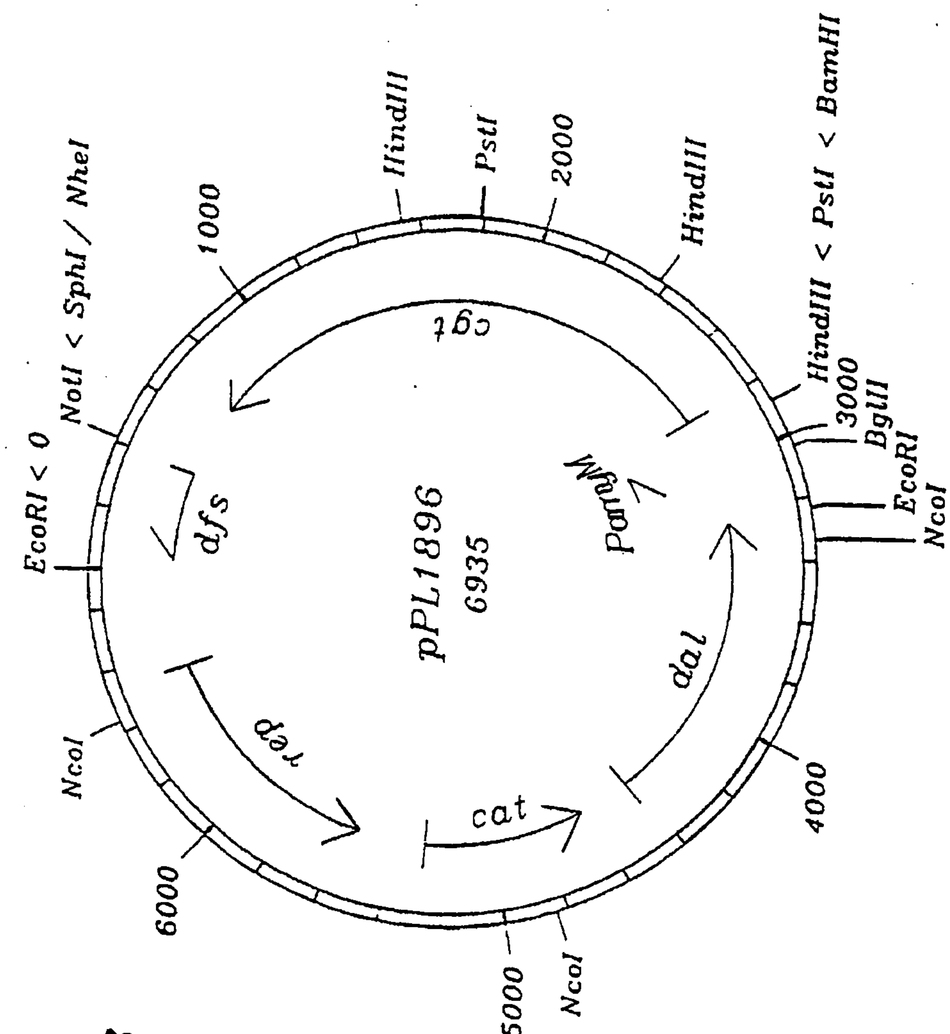

pPL1896
6935
1000
2000
3000
4000
5000
6000
HindIII
PstI
HindIII
HindIII < PstI < BamHI
BglII
EcoRI
NcoI
NcoI
NcoI
EcoRI < 0
NotI < SphI / NheI
cgt
PamyM
dal
cat
rep
dfs

FIG.23

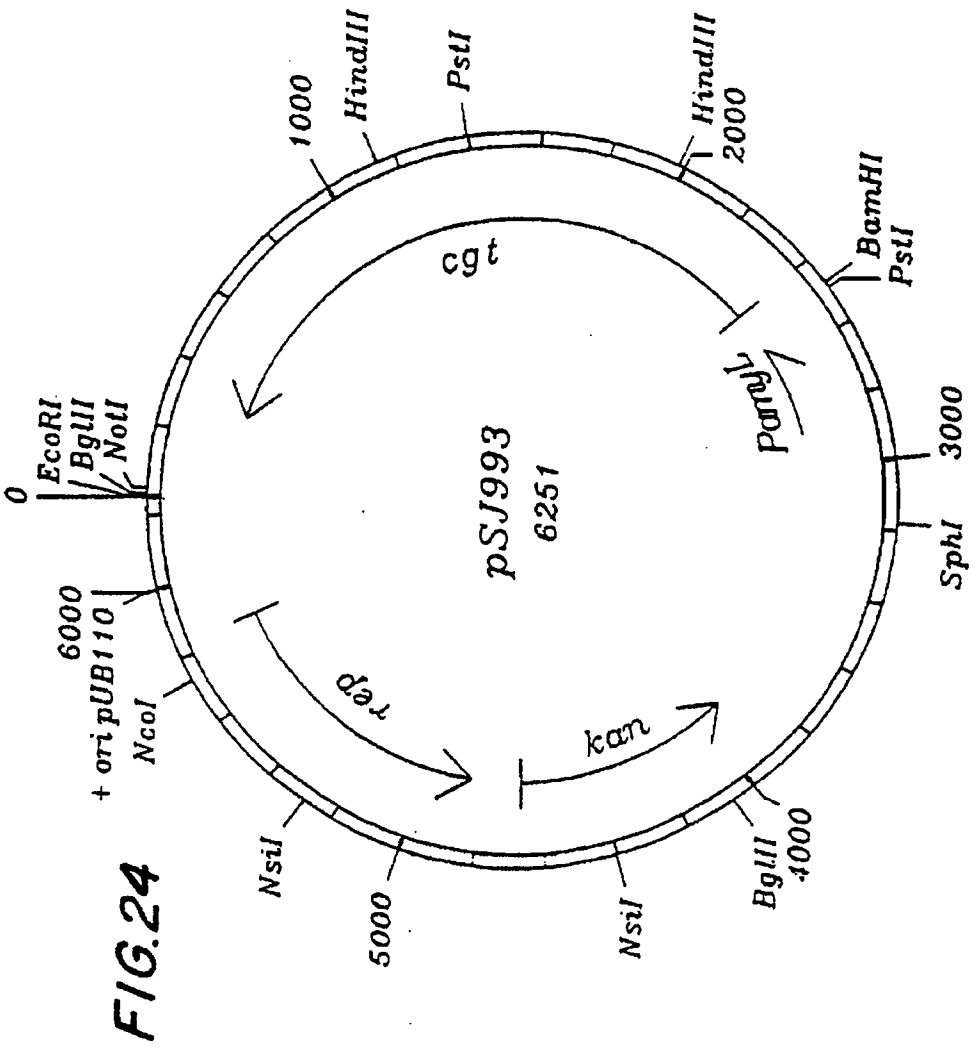

0
EcoRI
BglII
NotI
1000
HindIII
PstI
2000
HindIII
BamHI
PstI
3000
SphI
4000
BglII
NsiI
5000
NsiI
6000
+ ori pUB110
NcoI
cgt
pSJ993
6251
kan

FIG.24

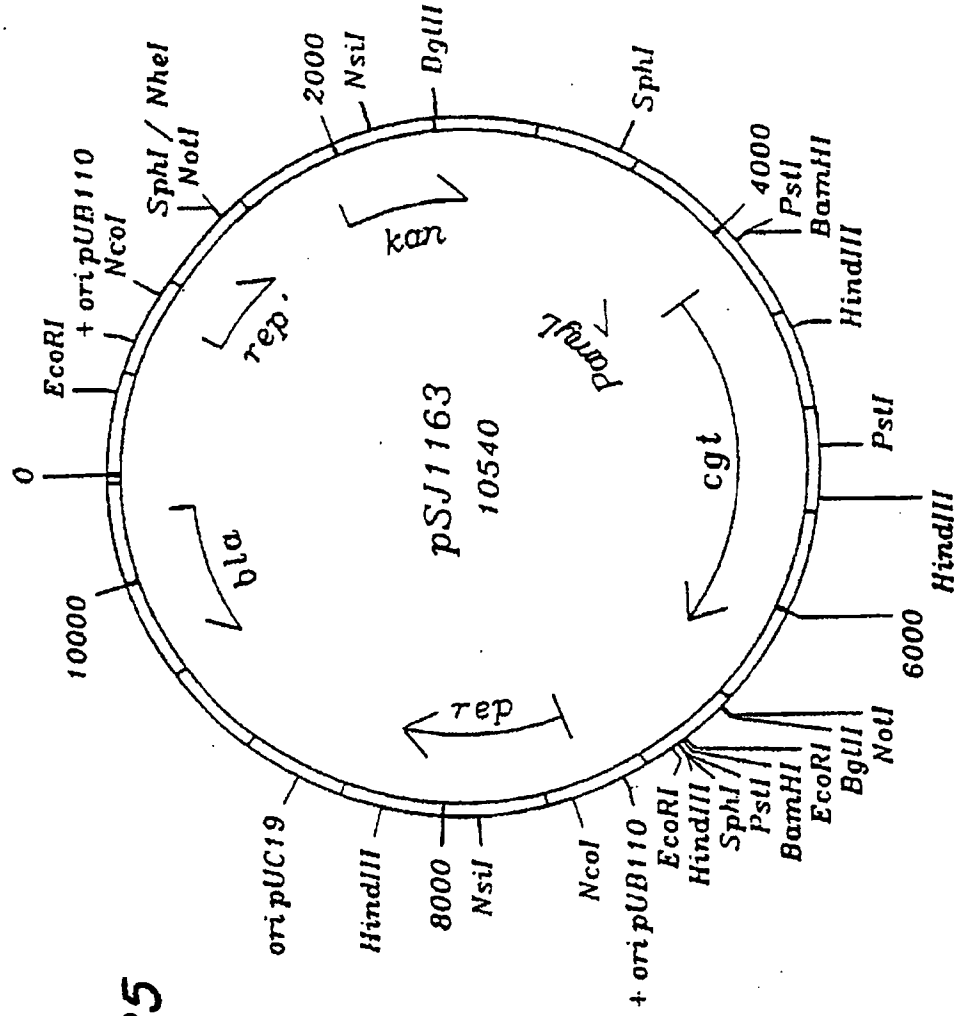

pSJ1163
10540
0
2000
4000
6000
8000
10000
EcoRI
+ ori pUB110
NcoI
SphI / NheI
NotI
NsiI
BglII
SphI
PstI
BamHI
HindIII
PstI
HindIII
BglII
NotI
EcoRI
PstI
BamHI
EcoRI
HindIII
SphI
EcoRI
+ ori pUB110
NcoI
NsiI
HindIII
ori pUC19
bla
rep.
kan
rep
pamyl
cgt

FIG. 25

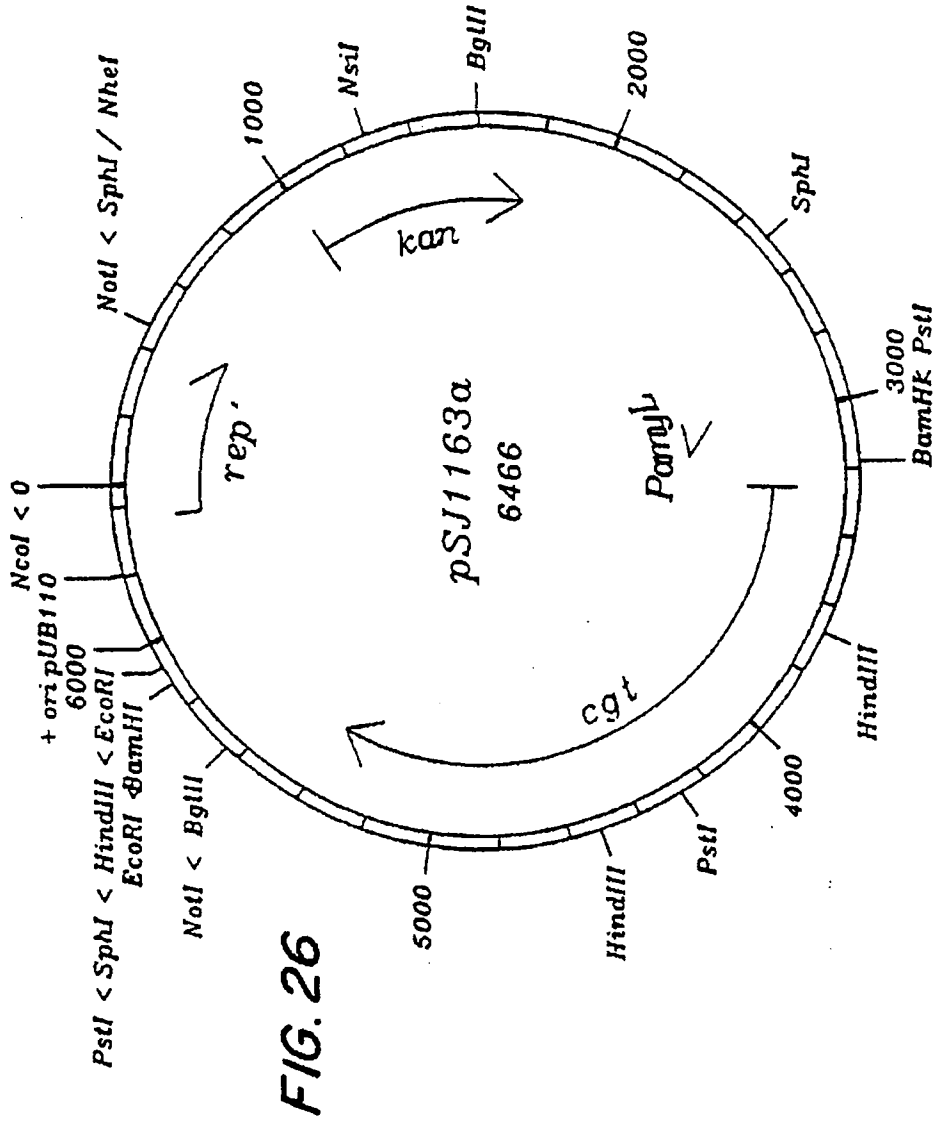

pSJ1163a
6466
kan
rep'
cat
Pamyl
NcoI < 0
+ ori pUB110
6000
PstI < SphI < HindIII < EcoRI
EcoRI BamHI
NotI < BglII
5000
HindIII
PstI
4000
HindIII
3000
BamHK PstI
SphI
2000
BglII
NsiI
1000
NotI < SphI / NheI

FIG. 26

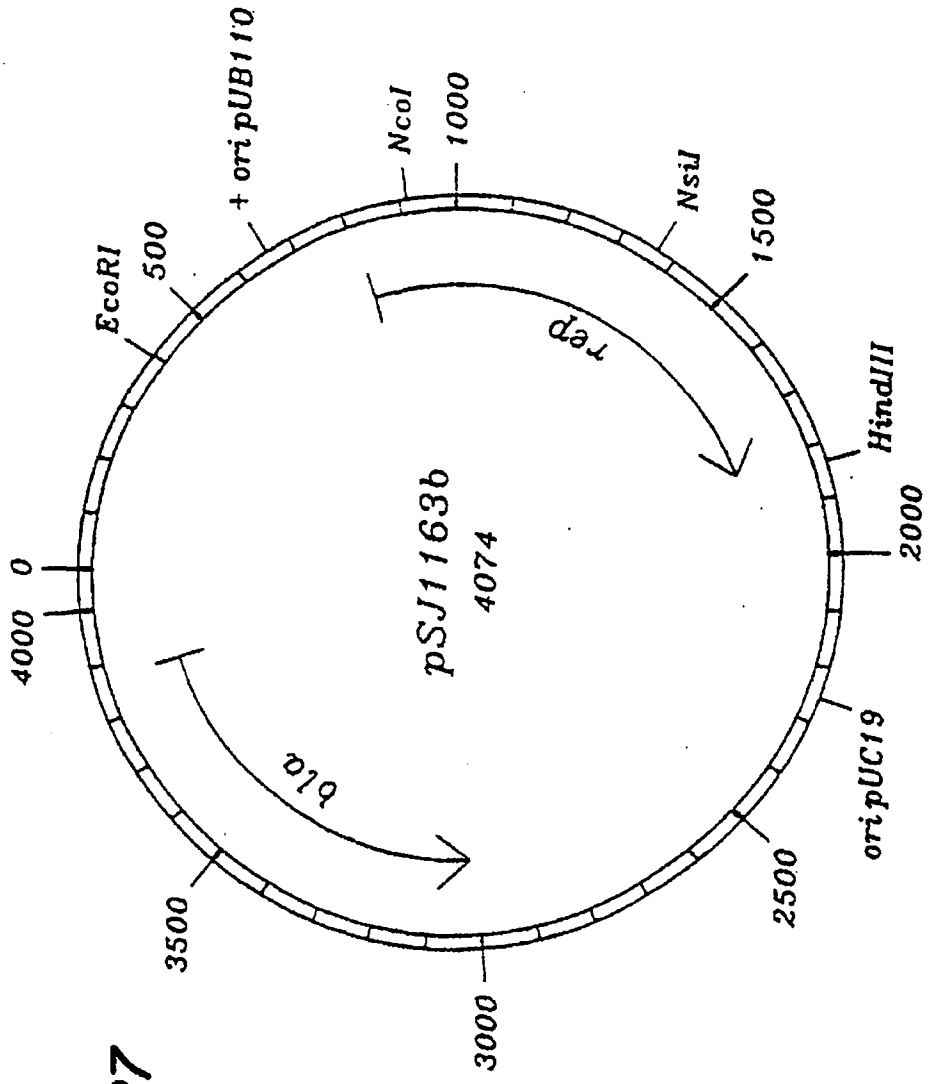

pSJ1163b
4074
0
500
1000
1500
2000
2500
3000
3500
4000
EcoRI
+ ori pUB110
NcoI
NsiI
HindIII
ori pUC19
rep
blo

FIG. 27

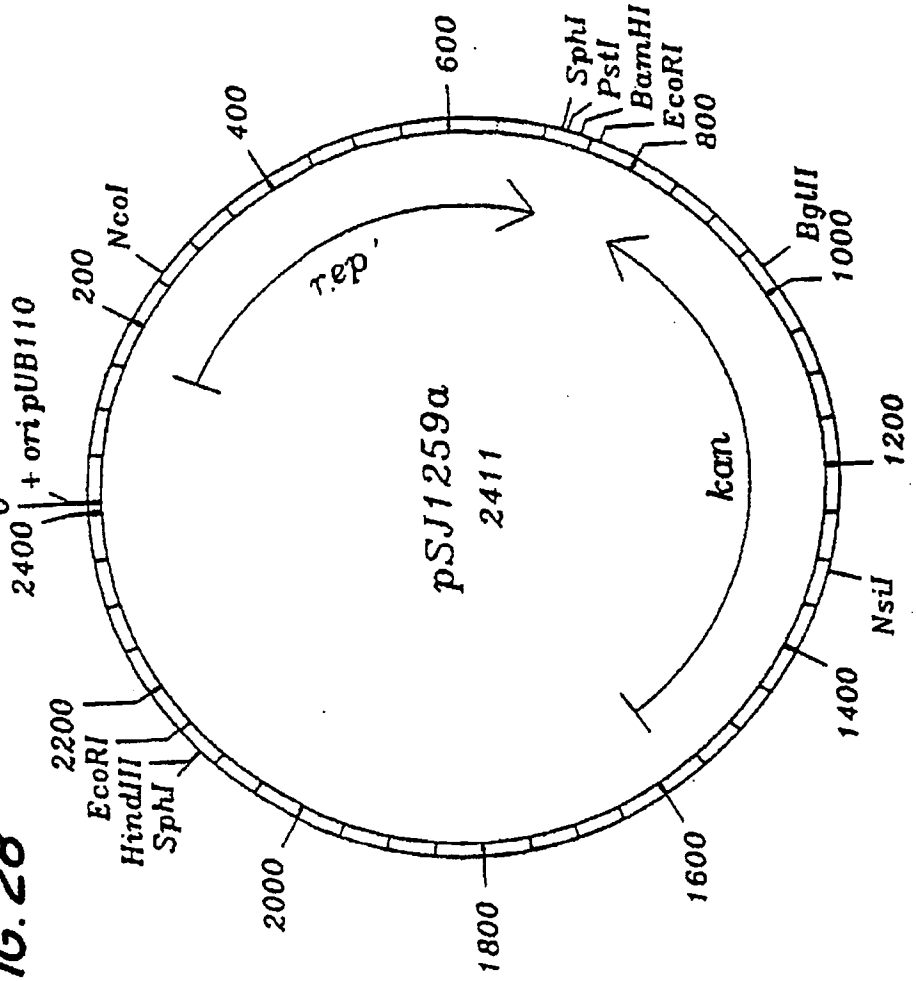

0
+ ori pUB110
200
NcoI
400
600
SphI
PstI
BamHI
EcoRI
800
BglII
1000
1200
NsiI
1400
1600
1800
2000
2200
EcoRI
HindIII
SphI
2400
rep
pSJ1259a
2411
kan

FIG. 28

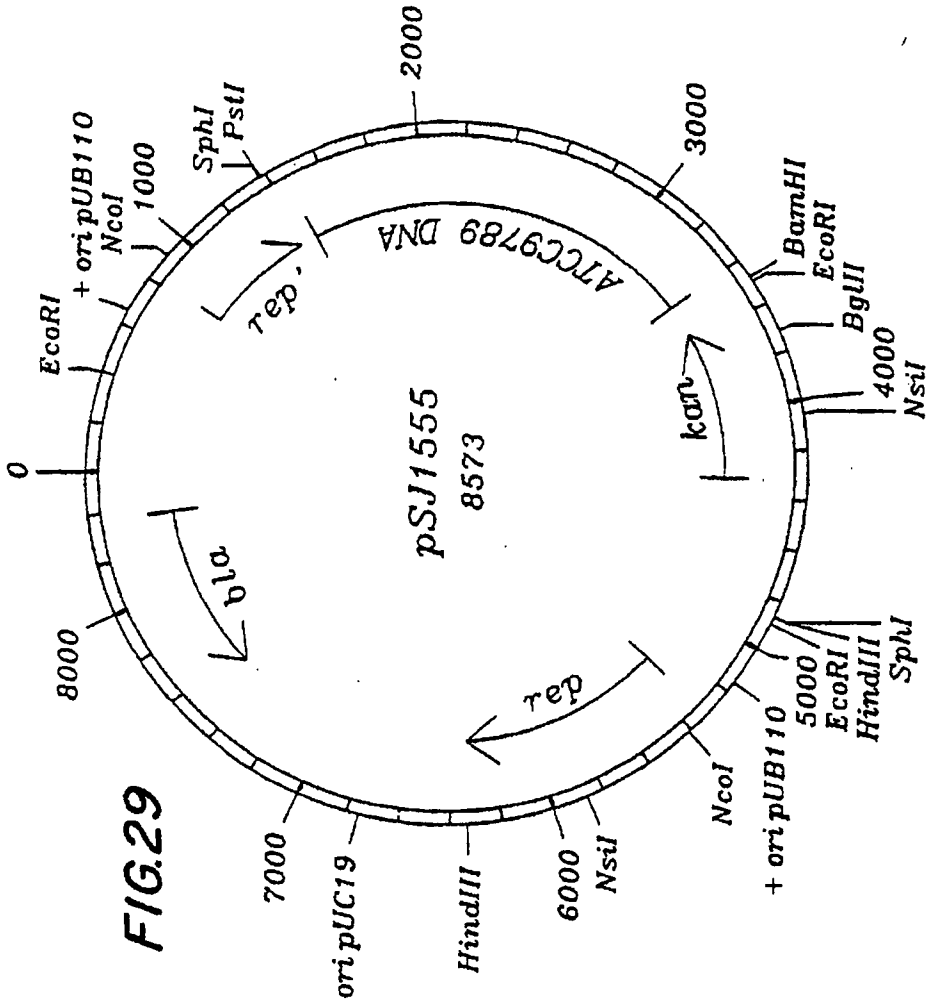

pSJ1555
8573
ATCC9789 DNA
rep.
kan
bla
rep
0
1000
2000
3000
4000
5000
6000
7000
8000
EcoRI
+ ori pUB110
NcoI
SphI
PstI
BamHI
EcoRI
BglII
NsiI
HindIII
SphI
NcoI
+ ori pUB110
NsiI
HindIII
ori pUC19

FIG.29

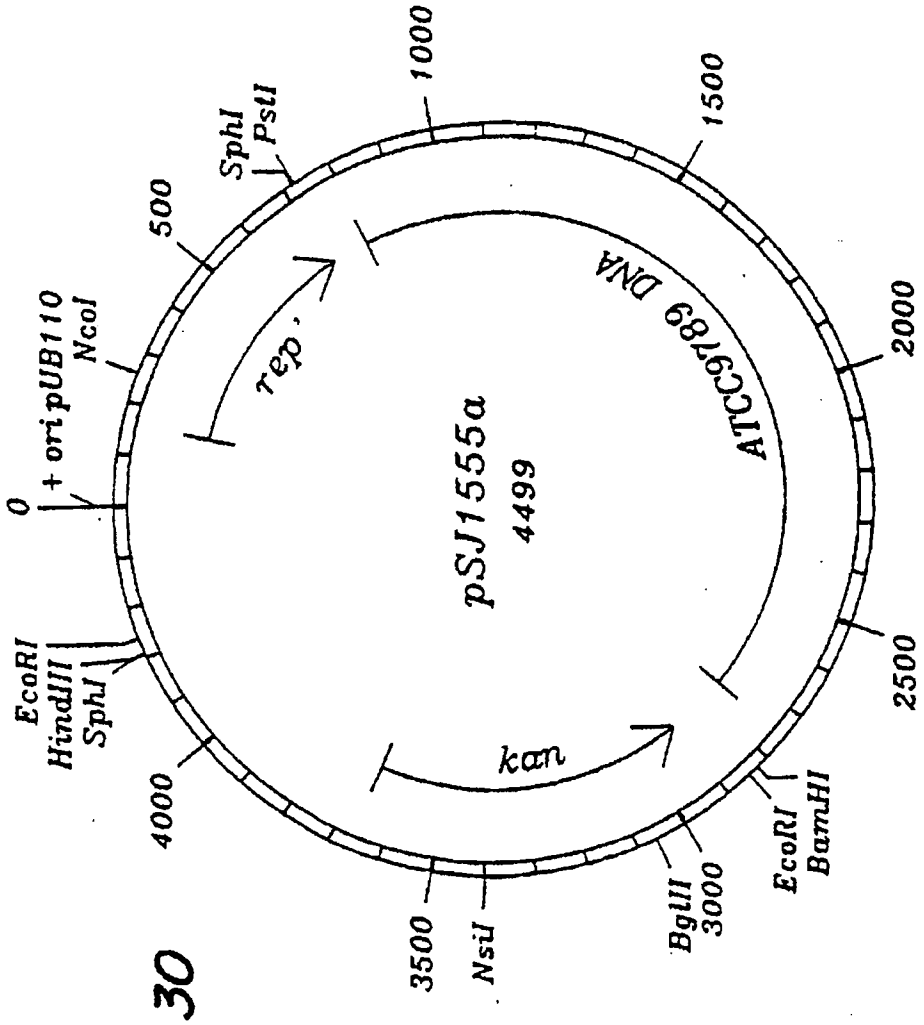

0
+ ori pUB110
NcoI
500
SphI
PstI
1000
1500
2000
2500
3000
BglII
EcoRI
BamHI
3500
NsiI
4000
EcoRI
HindIII
SphI
rep.
kan
ATCC6918 DNA
pSJ1555a
4499

FIG.30

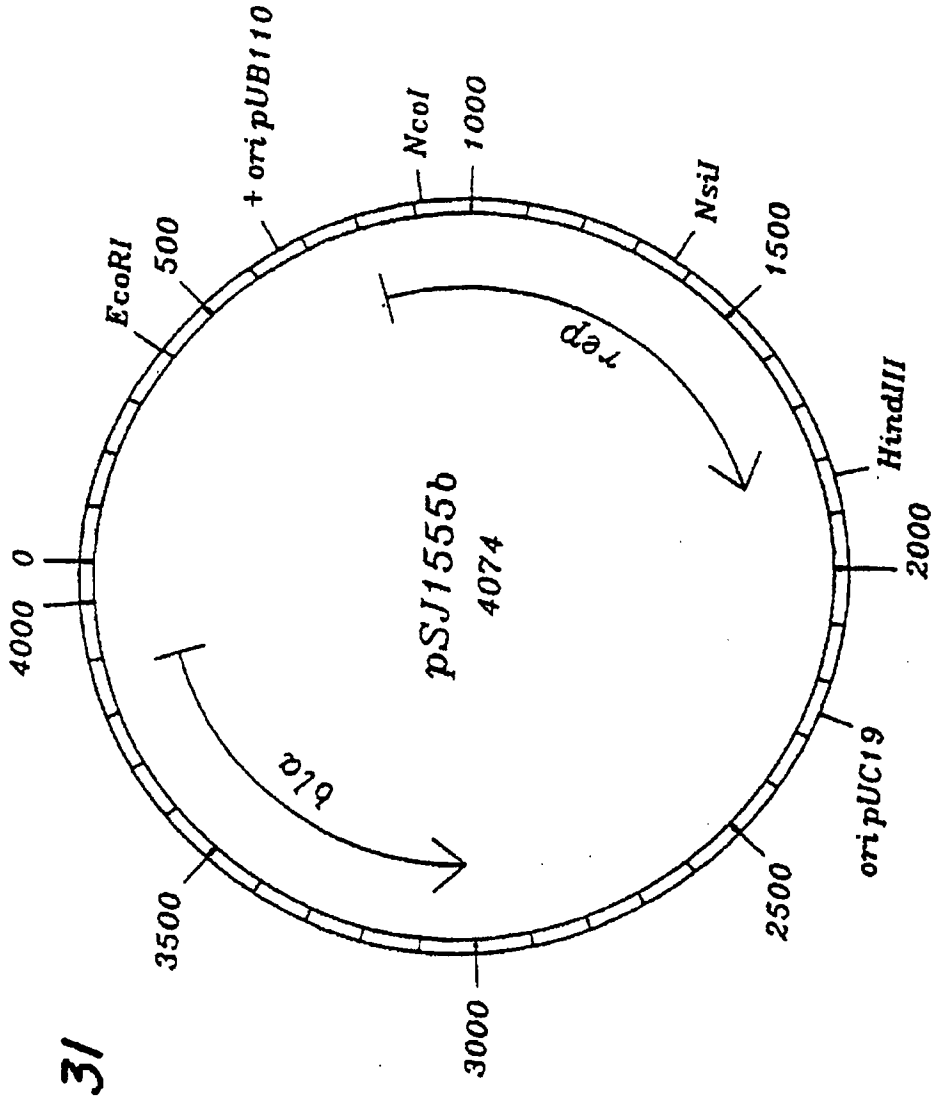

+ ori pUB110
NcoI
1000
NsiI
1500
HindIII
2000
ori pUC19
2500
3000
3500
4000
0
EcoRI
500
pSJ1555b
4074
tet
bla

FIG. 31

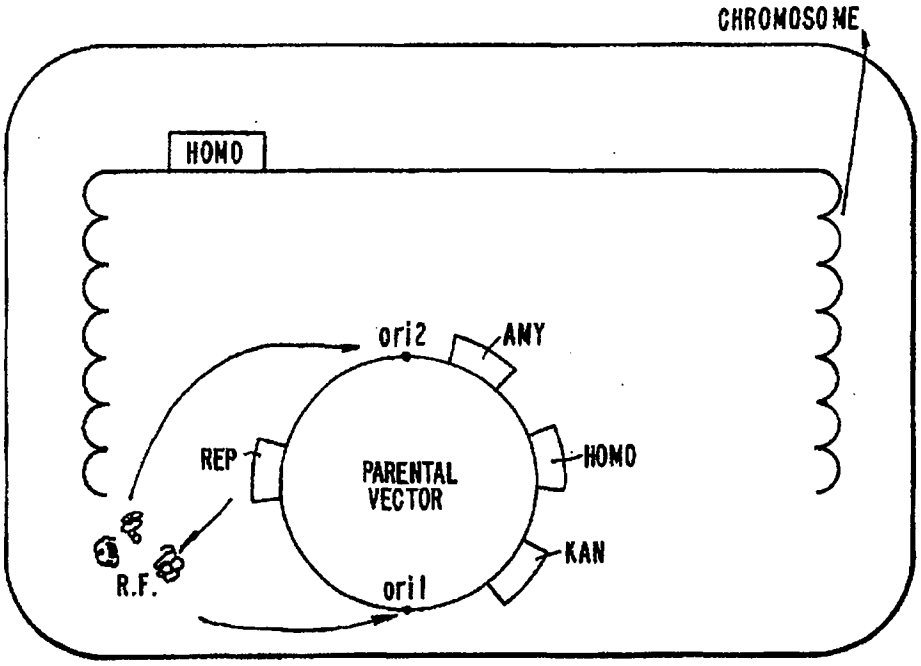
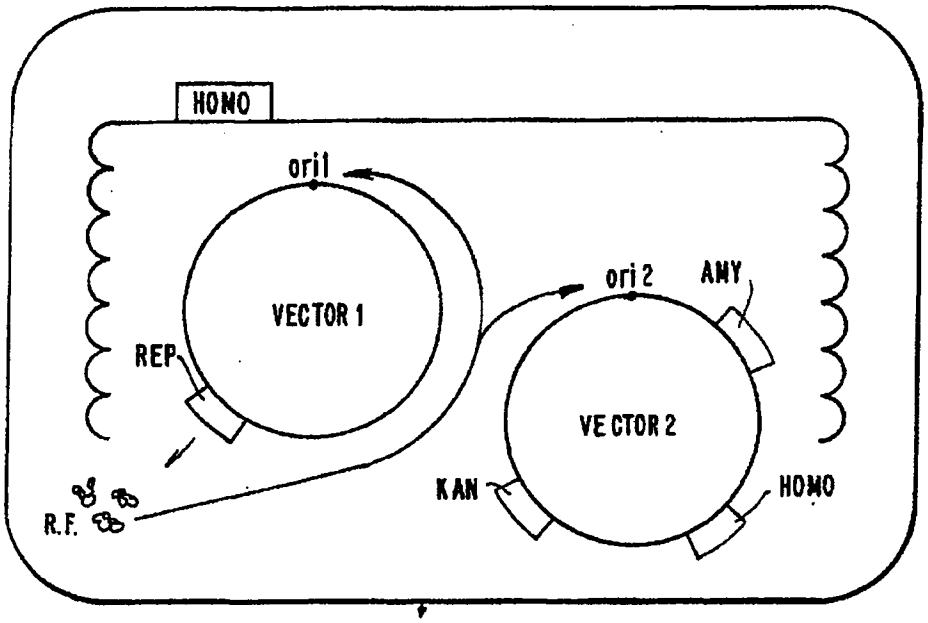


FIG. 32A

CULTIVATION → REPLICATION

FIG. 32B



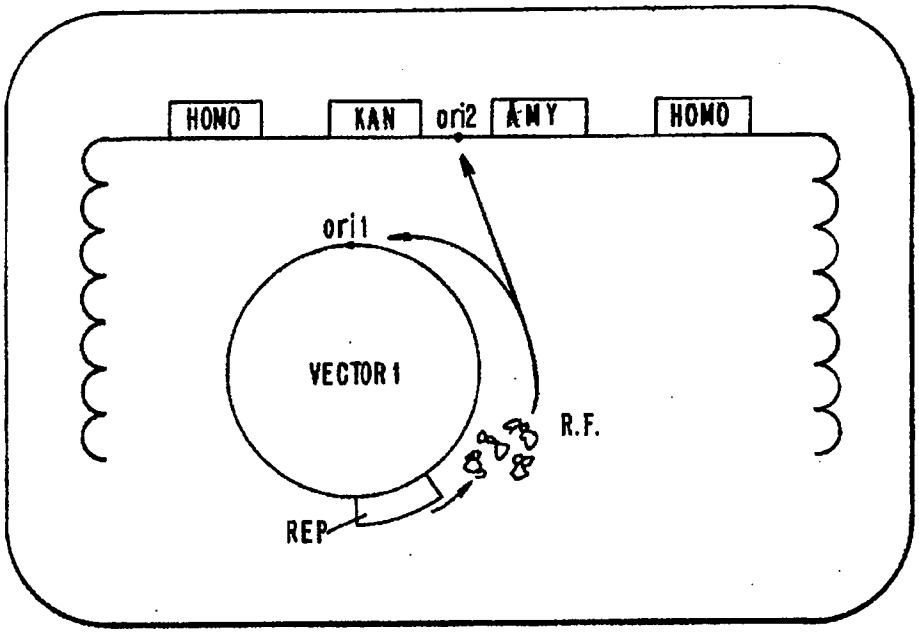
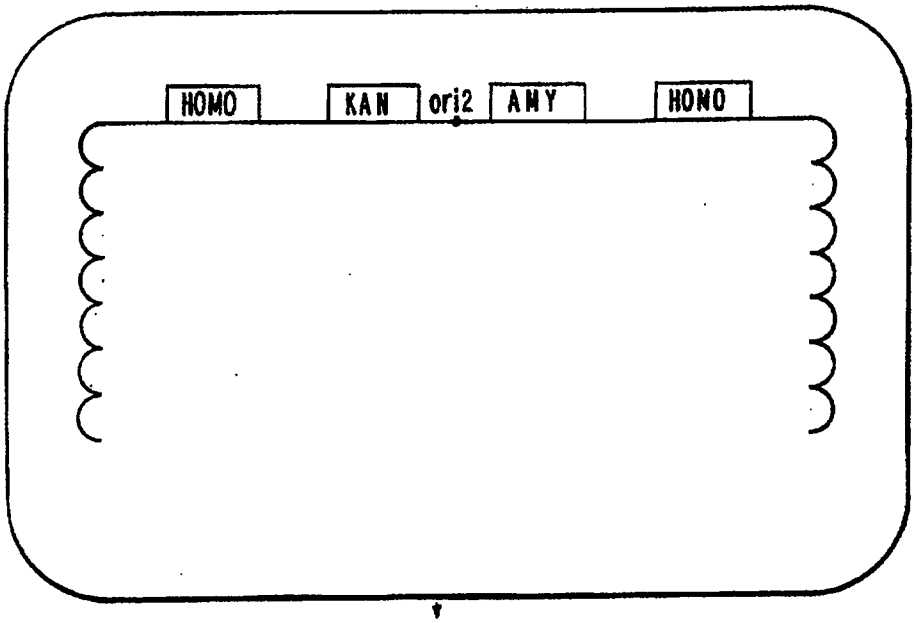


FIG. 32C

CULTURED WITH KANAMYCIN

FIG. 32D



STABLE INTEGRATION OF DNA IN BACTERIAL GENOMES

This application is a continuation of application Ser. No. 07/853,701, filed May 26, 1992, now abandoned, the contents of which are incorporated herein by reference.

FIELD OF INVENTION

The present invention relates to a bacterial cell which comprises a DNA construct integrated in its genome, a DNA construct intended for integration in the genome of a bacterial cell, a plasmid vector comprising the DNA construct, and methods of integrating the DNA construct into bacterial genomes.

BACKGROUND OF THE INVENTION

When, for the purpose of producing a desired polypeptide by recombinant DNA procedures, bacterial cells are transformed with a recombinant plasmid vector which carries inserted genetic information coding for said polypeptide, it has often been observed that such plasmids become unstable even though they may, in themselves, be stably inherited in the cell. This instability may either take the form of unstable maintenance of the plasmid in the cells so that the plasmid will eventually be lost from a cell population, or so that the DNA coding for the protein in question may be deleted from the plasmid. A traditional way of solving the former problem has been to grow the transformed cells under selection pressure, that is, typically in the presence of an antibiotic to which the cells in question have been made resistant due to the presence of a gene coding for a product mediating resistance to that antibiotic on the plasmid transformed to the cells. This approach, however, is neither economically feasible in large-scale production due to the high cost of the antibiotics in question, nor is it desirable for environmental reasons. The use of antibiotics in culture media also makes it more difficult to obtain product approval from health authorities and the like.

It has previously been suggested to stabilise plasmids by inserting into them a DNA sequence encoding a partitioning function which ensures the even distribution of plasmids to progeny cells on cell division. An alternative method of achieving the stable inheritance of cloned DNA sequences is to provide for the integration of such DNA sequences in the genome of the host bacterium. Integration of DNA sequences present on plasmid vectors may take place by the so-called "crossing-over" procedure, e.g. as described by A. Campbell, *Advances Genet.* 11, 1962, pp. 101–145. According to this procedure, the plasmid vector is provided with a DNA sequence which is homologous to a region on the bacterial genome, or alternatively with two homologous sequences placed on either side of the heterologous DNA sequence to be integrated. In a subsequent recombination event, the homologous sequence and adjacent sequences on the vector are integrated into the host genome at the region of homology.

In some cases, however, is has been found that the integrated DNA sequences are deleted from the cells in the absence of selection pressure, for instance by a similar type of homologous recombination event as that responsible for the integration of the DNA. In particular, it has previously been observed that recombination between homologous DNA sequences is stimulated in the proximity of replicative DNA present on or near the DNA integrated in the host cell genome, cf. Ph. Noirot et al., *J. Mol. Biol.* 196, 1987, pp. 39–48; and M. Young and S. D. Ehrlich, *J. Bacteriol.* 171(5), May 1989, pp. 2653–2656.

An object of the present invention is therefore to provide stable integration of DNA sequences into genomic DNA, e.g. the chromosome, of bacterial host cells.

SUMMARY OF THE INVENTION

The present invention is based on the finding that stable integration of DNA sequences into the genome of host bacteria may be obtained by avoiding the presence of a functional plasmid replication system in the integrated DNA.

Accordingly, in one aspect, the present invention relates to a bacterial cell which in its genome carries an integrated non-replicative DNA construct comprising (1) a DNA sequence of interest, (2) a DNA sequence which is homologous with a region of the genome of the cell, and (3) an origin of replication, said DNA construct lacking a functional gene coding for a factor required to initiate replication from said origin of replication.

In another aspect, the present invention relates to a DNA construct comprising (1) a DNA sequence of interest, (2) a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the DNA construct, and (3) an origin of replication, said DNA construct lacking a functional gene coding for a factor required to initiate replication from said origin of replication.

In the present context, the term "non-replicative DNA construct" is intended to mean a DNA sequence which is unable to replicate autonomously and which is therefore replicated together with the host cell genome. The genome comprises the chromosome and stably inherited extrachromosomal elements. The term "DNA sequence of interest" is used to indicate a sequence which may code for a desired RNA or protein product (heterologous or native to the host cell) or which may in itself provide the host cell with a desired property, e.g. a mutant phenotype as described below.

The homologous DNA sequence may typically be one derived from the genome of the host cell, and may be homologous with a region of the genome which is not essential for the survival or proper functioning of the host cell. On the other hand, the homologous DNA sequence may also be so selected that integration of the DNA construct of the invention by homologous recombination will lead to a cell expressing a mutant phenotype (which may then serve as a marker for selection of cells in which the DNA construct has been integrated), for instance if the DNA construct is integrated within a transcription unit disrupting this so that one or more gene(s) contained within the transcription unit are consequently not expressed. The homologous DNA sequence may alternatively be one which is not native to the host genome but which has been cloned from another organism or which has been synthesized and subsequently introduced into the host genome by any convenient process, e.g. crossing-over, prior to the integration of the DNA construct of the invention. The homologous DNA sequence may be one which comprises or consists essentially of the DNA sequence of interest, whether native or foreign to the host cell in question (for instance native to the cell in cases where it is desired to amplify the copy number of the DNA sequence of interest in the cell, vide below). It should ba noted that, in the present context, the term "homologous" may be defined as a sequence identity of at least 9 consecutive base pairs.

DETAILED DISCLOSE OF THE INVENTION

Although, according to the invention, the stable integration of DNA into bacterial genomes has been demonstrated

for plasmids with a particular type of replication system (the so-called rolling circle replication, vide below), it is currently expected that any plasmids which replicate by a mechanism where one or more trans-acting factors (i.e. RNA or protein factors) are required to initiate replication from cis-acting sequences on the plasmid (such cis-acting DNA sequences are collectively termed the origin of replication) will be useful for the present purpose. Factors which are necessary for plasmid replication will be termed replication factors in the following. When the DNA construct lacks a functional gene coding for a replication factor required by the origin of replication contained on said DNA construct, no active replication factor is produced and, consequently, no replication is initiated from the origin.

In order to obtain a DNA construct of the invention which lacks a functional gene encoding a required replication factor, one may either delete the entire gene or modify it in such a way that it encodes an inactive replication factor. Such modification of the gene may be carried out in a manner known per se by deletion, insertion or substitution of one or more nucleotides of the DNA sequence of the gene, or by similar modifications of transcriptional or translational start or stop signals.

The replication system outlined above may be utilised in a method of constructing a bacterial cell of the invention. In one embodiment of the method, a plasmid vector for the present purpose termed a parental vector, is initially constructed. The parental vector comprises (i) a first origin of replication; (ii) one or more functional genes encoding the replication factor(s) required for replication from said first origin of replication; (iii) a second origin of replication in the same orientation as the first origin of replication; (iv) a DNA sequence of interest, and (v) a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the vector, said parental vector lacking a functional gene encoding a replication factor required for replication from the second origin of replication in the region between the second and the first origin of replication (in the above-mentioned order). It should be noted that, in the present context, the term "plasmid" is also intended to denote a bacteriophage or other DNA molecule capable of functioning as an autonomously replicating extrachromosomal element.

According to the invention, this parental vector may then be transformed into bacterial cells, and the transformed cells are cultured under conditions permitting replication of the vector.

Replication of the parental vector in the transformed cell gives rise to the formation of two different progeny vectors.

The first progeny vector comprises (i) a first origin of replication; (ii) one or more genes encoding the replication factor(s) required for replication from said origin. The second progeny vector comprises (iii) a second origin of replication; (iv) a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the plasmid vector, said second progeny vector lacking a functional gene coding for a replication factor required for replication from the second origin of replication carried on said second progeny vector. The formation of two progeny vector molecules from the parent vector may take place by different mechanisms; either as a result of the mode of replication of the plasmid, for instance rolling circle replication of single-stranded DNA plasmids (vide below), or as a result of homologous recombination between the DNA regions including and/or adjacent to the two origins of replication present on the plasmid vector.

When the second origin is located in the same orientation on the parent plasmid as the first origin, the various DNA sequences intended to be integrated into the bacterial genome and located downstream of the second origin, but upstream of the first origin (i.e. the DNA sequence of interest, and the DNA sequence which is homologous with a region of the genome of the cell) will be present on the second progeny vector following plasmid replication. Continued culturing of the transformed cells may spontaneously result in the integration of said second progeny vector into the bacterial genome by homologous recombination and loss of the first progeny vector from the cells with a certain frequency.

In order to facilitate selection for cells in which the second progeny vector has been integrated in the genome, this vector is preferably provided with a selectable marker. In this case, the cells may be cultured under selective conditions which is to say that only cells in which the selectable marker is maintained will survive. The selectable marker may be a gene coding for a product which confers antibiotic resistance to the cell or which confers prototrophy to an auxotrophic strain (e.g. dal genes introduced in a dal strain; cf. B. Diderichsen in *Bacillus: Molecular Genetics and Biotechnology Applications*, A. T. Ganesan and J. A. Hoch, Eds., Academic Press, 1986, pp. 35–46). Cells surviving under these conditions will either be cells containing the parental plasmid vector or containing both progeny vectors formed upon replication of the parental vector in the cell, or cells in which the second progeny vector comprising the DNA construct of the invention has been integrated. It has surprisingly been found that the parental vector and the first progeny vector are eventually lost whereas the second progeny vector comprising the DNA construct of the invention is spontaneously integrated in the host genome at a high frequency.

If it is desired to improve the efficiency with which integration of the DNA construct takes place, one may utilise, as the parental vector, a plasmid which is able to replicate under certain (permissive) conditions and unable to replicate under other (non-permissive) conditions. The plasmid may, for instance, be one which is temperature-sensitive for replication. Thus, in an embodiment of the method of the invention, the parental vector is one which is unable to replicate at increased temperatures, which yet permit growth of the host cell. The bacterial cells are initially cultured at a temperature permitting plasmid replication and formation of the two progeny vectors and subsequently, after integration of the second progeny vector, comprising the DNA construct of the invention, into the bacterial genome may have taken place, cultured at a temperature which does not permit plasmid replication so that the first progeny vector as well as the parental vector are lost from the cells. The cultivation at the non-permissive temperature is conducted under selective conditions to ensure that only cells containing the integrated DNA construct, including an appropriate selectable marker, will survive.

Another way of increasing the efficiency of integration and subsequent loss of the first progeny vector from the cells may be to treat the cells transformed with the parental vector with a plasmid-curing agent, e.g. novobiocin (Gadd, I. et al., 1987. Zbl. Bakt. Hyg. A. 265, 136–145), after culturing the host cells under selective conditions as described above.

It may be possible to employ replication origins from two different plasmids on the same parental vector, provided that these are sufficiently similar to each other to be functional with the same replication factor(s) which should be able to initiate replication from both the first and the second origin

of replication. Alternatively, the plasmid vector should contain homologous regions in order to be able to undergo homologous recombination as described above. It is, however, preferred that the first origin of replication (and the gene coding for the replication factor associated therewith) is derived from the same plasmid as the second origin of replication in order to ensure that the replication mechanism on which the present invention is based will function optimally.

Out of practical considerations, it may be preferred to make the initial construction of the plasmid vector in an organism in which replication from the first and second origins of replication cannot be initiated or in which the rate of replication from these origins is very low. The plasmid vector may therefore be a shuttle vector provided with an additional origin of replication which makes the vector able to replicate in two different organisms. The additional origin of replication may, for instance, be one which is functional in *Escherichia coli*, this organism being well described and conventionally used for recombinant DNA experimentation and therefore suitable for constructing plasmids by recombinant DNA techniques. The shuttle vector may also comprise an additional selectable marker, e.g. an antibiotic resistance gene, for selection of the vector in *E. coli*. The additional origin of replication and selectable marker should preferably follow the first origin and/or the replication factor(s), but precede the second origin so that, on replication of the vector from the first and second origins, these additional sequences will be carried by the first progeny vector which is eventually lost from the bacterial cell transformed with the parental vector.

In an alternative method of producing the bacterial cell of the invention, host cells are transformed with a first DNA vector comprising a first origin of replication associated with a functional gene encoding a factor required for plasmid replication from said first origin of replication, and subsequently or simultaneously, by cotransformation, with a second DNA vector comprising a second origin of replication lacking an associated functional gene encoding a factor required for plasmid replication from the second origin of replication, as well as a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of the cell. The second DNA vector is also preferably provided with a selectable marker. The resulting cells containing the first and second DNA vectors are then cultured, preferably under selective conditions as described above, which eventually leads to integration of said second DNA vector into the bacterial genome by homologous recombination and loss of the first DNA vector for which there is no selection. As in the method described above initially employing a single plasmid vector, the first DNA vector may be one the replication of which is dependent on permissive and non-permissive conditions for culturing cells transformed with the vector. Thus in the method of the invention, the first DNA vector may be one which is unable to replicate at increased temperatures, which yet permit growth of the host cells, and the bacterial cells are initially cultured at a temperature permitting plasmid replication and subsequently, after integration of the second DNA vector into the bacterial genome, cultured at a temperature which does not permit plasmid replication so that the first DNA vector is lost from the cells and under selective conditions so that only cells in which the second DNA vector is integrated, are able to survive. Similarly, the transformed cells may be treated with a plasmid-curing agent as described above.

An intermediate formed in both of the methods of constructing a cell containing the integrated non-replicative DNA construct discussed above is a bacterial cell which comprises a first DNA vector comprising a first origin of replication associated with one or more functional gene(s) encoding the factor(s) required for plasmid replication from said first origin of replication, and a second DNA vector comprising a second origin of replication, as well as a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of the cell, said second vector lacking a functional gene coding for a replication factor required for replicatin from the origin of replication carried on said second vector.

In order to obtain an origin of replication on the second DNA vector which lacks a functional gene coding for a replication factor required for replication from said origin, it is possible to delete this gene from the vector or to modify it in the ways indicated above. In particular when using two different origins of replication, the first DNA vector may also be provided with a gene coding for a replication factor required to initiate replication from the second origin of replication. In this way, replication of the second DNA vector depends on the second replication factor produced from the first DNA vector, and the second vector becomes non-replicative when the first vector is lost from the cell. However, the first and second origin of replication may also be derived from the same plasmid, in which case only one gene coding for an intact replication factor is required on the first vector.

Although, for the purpose of the present invention, the bacterial cell into which the plasmid vector or the first and second DNA vector are transformed may be both gram-negative and gram-positive, it is preferably a cell of a gram-positive bacterium as it is generally easier to obtain extracellular expression of polypeptides from gram-positive organisms than from gram-negative ones. Thus, the bacterium may be of a strain belonging to the genus Bacillus or Streptomyces, in particular a strain of *Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus subtilis* or *Streptomyces lividans*.

The present invention is currently believed to be the only efficient method of providing stable homologous integration of DNA sequences of interest in genomes of bacteria which cannot be transformed by being made competent (or, at least, in which natural competence mechanisms have yet to be demonstrated), but which may be transformed by techniques including, for instance, protoplast formation or electroporation, e.g. certain strains of *Bacillus licheniformis* or *Bacillus lentus*. The present method is therefore of particular interest with respect to such organisms in which the transformation frequency is low, typically 10–50 transformants per μg of DNA (contrary to, e.g., transformation of competent *E. coli* or *B. subtilis* cells, where the number of transformants is typically on the order of 10^6–10^8 per μg of DNA), which makes the sucessful transformation and stable integration of DNA in these organisms particularly important.

In the bacterial cell of the invention, the DNA sequence of interest is advantageously one which codes for a polypeptide of interest, and the present invention consequently further relates to a process for producing a polypeptide of interest, comprising culturing a bacterial cell according to the invention containing an integrated DNA sequence which codes for said polypeptide under conditions conducive to the production of the polypeptide and recovering the resulting polypeptide from the culture. The polypeptide produced by the present process may be any polypeptide advantageously

produced in bacteria such as an enzyme, e.g. a protease, amylase or lipase.

DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

A large family of plasmids from gram-positive bacteria replicate by the so-called "rolling circle replication" mechanism generating single-stranded DNA as a replication intermediate. Replication is initiated when a plasmid-encoded protein, Rep, recognizes an origin of replication sequence (the plus origin) and produces a nick in one of the DNA strands (the plus strand). The plus strand is then displaced, and a new plus strand is polymerized from the nick by 3'-OH extension. When the Rep protein subsequently recognizes a termination sequence (which overlaps the plus origin), it produces a second nick at the same position as the first one to generate a fully replicated strand and a single-stranded DNA monomer of the displaced strand the ends of which are ligated to form a circular molecule. Host factors then ensure the conversion of the single-stranded DNA molecule to double-stranded DNA (for a more detailed description of this type of plasmid, see A. Gruss and S. D. Ehrlich, *Microbiological Reviews* 53(2), June 1989, pp. 231–241). For the present purpose, plasmids with this replication system are termed single-stranded DNA plasmids.

It has surprisingly been found that the rolling circle replication mechanism may be utilised according to the invention to produce a bacterial cell according to the invention which harbours a DNA construct comprising, apart from a DNA sequence of interest and a DNA sequence which is homologous to a region of the genome of the cell, a plus origin of replication from a single-stranded DNA plasmid, lacking a functional rep gene cognate to the plus origin of replication.

This bacterial cell may be constructed by the method of the invention using a parental plasmid vector which comprises (i) a first plus origin from a single-stranded DNA plasmid; (ii) a functional red gene cognate to the first plus origin; (iii) a second plus origin from a single-stranded DNA plasmid in the same orientation as the first plus origin (iv) a DNA sequence of interest, and (v) a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the plasmid vector, said parental vector lacking, in the region between the second and the first plus origin in the same orientation as above, a functional red gene cognate to the second plus origin.

On replication of the parental vector, the first and second progeny DNA vectors are formed, presumably by the following mechanism:

The Rep protein initiates replication by producing a nick at the first plus origin, and proceeds to make a nick at the second plus origin. The displaced strand is religated to form a first progeny vector comprising a first plus origin of replication from a single-stranded DNA plasmid and a functional red gene cognate to the first plus origin. Similarly, the Rep protein proceeds from the second plus origin to make a nick at the first plus origin thus forming, on religation of the displaced strand and conversion of the single-stranded DNA to double-stranded DNA, a second progeny vector comprising a second plus origin of replication from a single-stranded DNA plasmid lacking a functional rep gene cognate to the second plus origin of replication, as well as a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of said cell. As the second progeny vector does not comprise a functional rep gene, replication of this molecule depends entirely on the Rep protein supplied in trans from either the first progeny vector or from the parental vector.

Alternatively, the two progeny vectors could also be formed as a result of recombination between the homologous DNA regions including and/or adjacent to the two origins present on the parental vector.

A second progeny vector without a functional replication origin may be formed if the first plus origin in the parental plasmid described above is replaced by a small DNA fragment derived from the origin region which is sufficient to ensure termination of plasmid replication but too small to constitute a functional origin. Such fragments have been identified in pUB110 (Boe et al., 1989, J. Bacteriol., 171, 3366–3372) and in pC194 (Gros et al., 1987, EMBO J., 6, 3863–3869).

The bacterial cell may alternatively be constructed by a method of the invention comprising transforming the host cell with a first DNA vector comprising a first plus origin of replication from a single-stranded DNA plasmid associated with a functional rep gene, and subsequently or simultaneously, by cotransformation, transforming the host cell with a second DNA vector comprising a second plus origin of replication from a single-stranded DNA plasmid lacking a functional rep gene cognate to the second plus origin of replication, but comprising a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of said cell. The second DNA vector is maintained in the cell due to the presence of Rep protein supplied from the first DNA vector.

When the parental vector or second DNA vector comprises a modified rep gene, the second plus origin may precede or be located in the modified rep gene. As described above, the second plus origin may be derived from the same or a different plasmid as the first plus origin. In cases where the first and second plus origins are derived from different plasmids so that replication will not be initiated from both origins by means of the same Rep protein, the first DNA vector may additionally contain a rep gene encoding an active Rep protein capable of initiating replication from the second plus origin. The parental vector or second DNA vector may also comprise a selectable marker as described above.

In a favoured embodiment for promoting the integration process, a vector may be employed the replication of which is dependent on permissive conditions, including the temperature at which host cells are cultured. Thus, when a host bacterium containing the first and second DNA vectors is cultured at the permissive temperature for plasmid replication, the Rep protein produced from the first DNA vector will serve to maintain the second DNA vector in the cell. However, at non-permissive temperatures at which the first DNA vector is unable to replicate, the first vector and consequently the Rep protein produced from it will be lost from the cell so that the second DNA vector is no longer able to replicate either. By continued cultivation under selection pressure, e.g. in the presence of an antibiotic, only those cells survive which in their genome contain the inserted DNA construct of the invention, including a gene coding for a selectable marker.

It should be noted that once the DNA construct has been integrated in the genome of the host cell, this may be cultured in the absence of selection pressure without consequent loss of the DNA construct or parts thereof from the cell. This is believed to be ascribable to the fact that the integrated DNA is incapable of autonomous replication, but is replicated together with the host genome. The lack of

autonomous replication of the integrated DNA implies that there is no formation of the single-stranded DNA intermediate which is believed to be responsible for the recombination process whereby integrated DNA is excised from the host genome (cf. Ph. Noiret et al., *J. Mol. Biol.* 196, 1987, pp. 39–48; and M. Young and S. D. Ehrlich, *J. Bacteriol.* 171(5), May 1989, pp. 2653–2656).

It has been found possible to amplify the integrated DNA construct by culturing transformed cells under increased selection pressure, e.g. at increased concentrations of an antibiotic. It has previously been found (cf.) that in the absence of selection pressure such amplified copies are frequently lost from the cells. Contrary to this, the present invention provides a bacterial cell in which amplified copies of integrated DNA sequences may be stably maintained in host cells because, as explained above, the integrated DNA is non-replicative. Although the present invention has mainly been described above as suitable for the integration of heterologous DNA sequences, it should be noted that the present method is also suitable for obtaining an amplified copy number of a gene which is homologous to the host cell in order to increase its production of a specific gene product.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is further illustrated with reference to the accompanying drawings wherein

FIG. 1 shows a restriction map of plasmid pDN3000,

FIG. 2 shows a restriction map of plasmid pE194,

FIG. 3 shows a restriction map of plasmid pPL1975,

FIG. 4 shows a restriction map of plasmid pSX120,

FIG. 5 shows a restriction map of plasmid pPL2002,

FIG. 6 shows a restriction map of plasmid pDN3060,

FIG. 7 shows a restriction map of plasmid pSJ1085,

FIG. 8 shows a restriction map of plasmid pUC19,

FIG. 9 shows a restriction map of plasmid pSJ1103,

FIG. 10 shows a restriction map of plasmid pSJ1130,

FIG. 11 shows a restriction map of plasmid pSJ1136,

FIG. 12 shows a restriction map of plasmid pSJ1137,

FIG. 13 shows a restriction map of plasmid pPL1484,

FIG. 14 shows a restriction map of plasmid pSJ1155,

FIG. 15 shows a restriction map of plasmid pSJ1157,

FIG. 16 shows a restriction map of plasmid pSJ1259,

FIG. 17 shows a restriction map of plasmid pDN2904,

FIG. 18 shows a restriction map of plasmid pSJ1139,

FIG. 19 shows a restriction map of plasmid pSJ1139a,

FIG. 20 shows a restriction map of plasmid pSJ1139b,

FIG. 21 shows a restriction map of plasmid pdn3020,

FIG. 22 shows a restriction map of plasmid pPL1878,

FIG. 23 shows a restriction map of plasmid pPL1896,

FIG. 24 shows a restriction map of plasmid pSJ993,

FIG. 25 shows a restriction map of plasmid pSJ1163,

FIG. 26 shows a restriction map of plasmid pSJ1136a,

FIG. 27 shows a restriction map of plasmid pSJ1163b;

FIG. 28 shows a restriction map of plasmid pSJ1259a;

FIG. 29 shows a restriction map of plasmid pSJ1555;

FIG. 30 shows a restriction map of plasmid pSJ1555a; and

FIG. 31 shows a restriction map of plasmid pSJ1555b.

FIG. 32A shows a cell transformed with a single plasmid vector which has a first origin of plasmid replication (ori1) followed by the gene coding for the replication factor (rep), followed by a second origin of replication (ori2), a DNA sequence of interest (e.g. amy), and a selection marker (e.g. kan). FIG. 32B shows the segregation of the plasmid of FIG. 32A on replication. FIG. 32C shows the host cell containing the integrated DNA. FIG. 32D shows the host cell containing the integrated DNA after selection with kanamycin.

In all figures, arrows denote the direction of transcription.

To improve readability the replicational origins (+ori pUB110, +ori pE194, ori pUC19) are indicated by the actual start site for replication, even though a functional origin consists of a larger DNA region.

The invention is further described in the following examples which are not intended to be in any way limiting to the scope and spirit of the invention as claimed.

MATERIALS AND METHODS

Plasmids

pBD64: described in Gryczan et al., 1980.

pDN3060: A cloning vector derived from the Bacillus plasmid pDN1050 (Diderichsen, B., 1986) by insertion of synthetic oligonucleotides containing a number of useful restriction sites. The restriction map is shown in FIG. 6.

pDN2904: A derivative of the Bacillus plasmid pUB110 (Gryczan et al., 1978), containing both a chloramphenicol resistance gene and a kanamycin resistance gene. The restriction map is shown in FIG. 17.

pPL1484: A pUC19 (Yanisch-Perron et al., 1985) derivative containing a modified polylinker region into which was inserted a 1.4 kb BamHI fragment from pDN2904 containing the kanamycin resistance gene. The restriction map is shown in FIG. 13.

pPL1878: pDN1380 (described in Diderichsen and Christiansen, 1988) containing a 2.4 kb HaeII-SphI fragment encoding a Cyclodextrin Glycosyl Transferase (CGTase) originating from Thermoanaerobacter sp. ATCC 53627. The gene was initially cloned into the *E. coli plasmid* pBR322 on a 12.8 kb EcoRI fragment (Starnes et al., 1989). The restriction map is shown in FIG. 22.

Strains

E.coli SJ 6: a restriction-deficient derivative of MC1000 (Diderichsen et al., 1990) *Bacillus subtilis* DN1885: an amyE, amyR2, spo$^+$, Pro$^+$ derivative of *B. subtilis* 168. (Diderichsen et al., 1990).

Bacillus subtilis DN1686:A spo derivative of DN1280 containing a chromosomal deletion in the dal gene (Diderichsen, 1986).

Bacillus licheniformis ATCC 9789

Bacillus lentus NCIB 10309

Media

TY:	Trypticase	20 g/l
	Yeast extract	5 g/l
	$FeCl_2.4H_2O$	6 mg/l
	$MnCl_2.4H_2O$	1 mg/l
	$MgSO_4.7H_2O$	15 mg/l
	pH	7.3
TY9:	As TY media but the pH was adjusted to 8,5 by adding $NaHCO_3$ (0,1M)	
TY9 agar:	Trypticase	20 g/l
	Yeast extract	5 g/l
	$FeCl_2.4H_2O$	6 mg/l

-continued

	$MnSO_4.4H_2O$	1 mg/l
	$MgSO_4.7H_2O$	15 mg/l
	Bacto agar	5 g/l
	Adjusted to pH 8,5 with $NaHCO_3$ (0,1M)	
BPX:	Potato starch	100 g/l
	Barley flour	50 g/l
	BAN 5000 SKB	0.1 g/l
	Sodium caseinate	10 g/l
	Soy Bean Meal	20 g/l
	Na_2HPO_4, 12 H_2O	9 g/l
	Pluronic	0.1 g/l
LB agar:	Bacto-tryptone	10 g/l
	Bacto yeast extract	5 g/l
	NaCl	10 g/l
	Bacto agar	15 g/l
	Adjusted to pH 7.5 with NaOH	

GENERAL METHODS

The experimental techniques used to construct the plasmids were standard techniques within the field of recombinant DNA technology, cf. T. Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N.Y., 1982.

Restriction endonucleases were purchased from New England Biolabs and Boehringer Mannheim and used as recommended by the manufacturers. T4 DNA ligase was purchased from New England Biolabs and used as recommended by the manufacturer.

Preparation of plasmid DNA from all strains was conducted by the method described by Kieser, 1984.

Transformation of *E. coli*

Cells of *E. coli*, were made competent and transformed as described by Mandel and Higa, 1970, or were transformed by electroporation as described in the manual for the BIO-RAD Gene Pulser electroporation apparatus.

Transformation of *B. subtilis*

Competent cells were prepared and transformed as described by Yasbin et al., 1975.

Transformation of *B. licheniformis*

Plasmids were introduced into *B. licheniformis* by polyethylene glycol-mediated protoplast transformation as described by Akamatzu, 1984.

Transformation of *B.lentus*

Plasmids were introduced into *B.lentus* by protoplast transformation according to a slightly modified procedure by Akamatzu (1984). The modifications were a higher pH in the regeneration medium e.g. the HCP 1,5 medium were buffered to pH 8,5 by adding 0,1M $NaHCO_3$ to the medium.

EXAMPLE 1

Stable integration of a non-replicative DNA molecule in the *Bacillus lentus* chromosome.

Cloning of the Subtilisin 309 gone

The gene coding for the protease designated subtilisin 309 was cloned from an isolate of the *B.lentus* strain NCIB 10309 as described in WO 89/06279. Further subcloning resulted in the plasmid pSX120, which contains the replication origin of pUB110, the chloramphenicol resistance gene (cat) from pC194, two promoters P_{AmyM} and P_{AmyQ} and the gene encoding for the subtilisin 309 protease. (See FIG. 4 and International Patent Application No. PCT/DK90/00164)

Construction of the integration plasmid pPL2002.

Plasmid pDN3000 was constructed by restricting pUC19 (Yanisch-Perron et. al.) with EcoRI and inserting the following oligonucleotide sequence (prepared by the phosphoamidite method described by Beaucage and Caruthers, *Tetrehedron letters* 22, 1981, pp. 1859–1869, on an automatic DNA synthesizer) (SEQ ID NO:1)

AATTGATCAAGCTTTAAATGCATGCTAG-
CAACGCGGCCGCCAACCTCGAGATCTCATG
CTAGTTCGAAATTTACGTACGATCGT-
TGCGCCGGCGGTTGGAGCTCTAGAGTACTTAA

into the linearized pUC19 followed by ligation. The ligation mixture was then used to transform competent *E.coli* SJ6 cells and transformants were selected on LB plates containing 100 ug/ml ampicillin. The orientation of the inserted linker in pDN3000 is as indicated by the orientation of the restriction sites in FIG. 1.

Plasmid pPL1975 was constructed by restricting pDN3000 with BglII followed by ligation of this linearized plasmid to the MboI fragment containing the DNA from position 1 to 1585 resulting from restriction of pE194 (FIG. 2, Horinouchi and Weisblum) with MboI. The ligation mixture was then used to transform competent *E.coli* SJ6 cells and transformants were selected on LB plates containing 100 ug/ml ampicillin. The orientation of the connection of these two fragments is as indicated in FIG. 3. pPL1975 thus contains a functional *E. coli* replication origin and a pE194 DNA fragment comprising an intact plus origin (+ ori pE194) and a truncated repF gene (repF') (Villafane et al., 1987).

Plasmid pPL2002 (FIG. 5) was constructed by restricting pPL1975 (FIG. 3) by EcoRI and BamHI and ligating the linearized plasmid to the 3.3 kb EcoRI (partial), BglII fragment from pSX120 (FIG. 4) containing the subtilisin 309 gene and the cat resistance gene. The ligation mixture was then used to transform competent *E. coli* SJ6 cells and transformants were selected on LB plates containing 100 ug/ml ampicillin.

Stable integration of the pPL2002 plasmid into the chromosome of *B.lentus*.

An isolate of the *B.lentus* strain NCIB 10309 was transformed by protoplast transformation with the temperature sensitive plasmid pE194 (See FIG. 1) selecting for erythromycin resistance (5 ug/ml) at 30° C. (permissive temperature). The resulting strain was denoted PL2156.

PL2156 was then protoplast transformed with the plasmid pPL2002 selecting for chloramphenicol resistance (8 ug/ml) and erythromycin resistance (5 ug/ml) at 30° C. resulting in the strain PL2157 containing the two plasmids pE194 and pPL2002. In these cells the replication of the plasmid pPL2002 completely depends on the presence of the plasmid pE194 which encodes the for the pPL2002 replication, indispensable replication protein repF.

The strain PL2157 was grown overnight in TY9 medium and dilutions were plated on TY9 plates at 45° C. (nonpermissive temperature) selecting for chloramphenicol restance (10 ug/ml).

One of these chloramphenicol resistant colonies was denoted PL2158

Southern hybridization showed that, in the strain PL2158, the plasmid pPL2002 was integrated into the chromosome by homologous recombination between the plasmid-borne and chromosomal subtilisin 309 genes and thereafter amplified to approx. 4 copies. No evidence of complete pE194 plasmid sequences was detected.

The stability of the chromosomally integrated copies of pPL2002 in strain PL2158 was tested in large scale fermentations (1500 l) without any antibiotic.

After fermentation samples were diluted and plated on TY9 plates and 100 colonies were replicated to TY9 plates

containing 10 ug/ml chloramphenicol. 98 of the tested colonies were still resistant to chloramphenicol indicating that the plasmid pPL2002 was still integrated in the chromosome. 20 of these colonies were then tested by Southern hybridisation which showed that the plasmid pPL2002 was still integrated, apparently in the same copy number (approx. 4 copies) in all of the tested colonies.

EXAMPLE 2

Construction of plasmid vectors containing two pUB110 origins of replication

Plasmid pSJ1085 (FIG. 7) was constructed by restricting pDN3060 (containing an origin of replication (+ ori pUB110) and rep gene (rep) from pUB110, and a chloramphenicol resistance gene (cat) from pC194) with BamHI and EcoRI and inserting the following oligonucleotide sequence (prepared by the phosphoamidite method described by Beaucage and Caruthers, *Tetrahedron Letters* 22, 1981, pp. 1859--1869, on an automatic DNA synthesizer) (SEQ ID NOS:2 and 3)

AATTCTGCAGATATCAAGATAAGAAA-
GAACAAGTTCCG

GACGTCTATAGTTCTATTCTTTCTTGT-
TCAAGGCCTAG

into the linearized pDN3060 followed by ligation and transformation of *B. subtilis* DN1885.

Plasmid pSJ1103 (FIG. 9) was constructed by restricting pSJ1085 (FIG. 7) with EcoRI and inserting the entire linearized plasmid into the similarly restricted plasmid pUC19 (FIG. 8) followed by ligation and transformation of *E. coli* SJ6. The resulting plasmid pSJ1103 contains the plus origin and rep gene from pUB110, the cat gene from pC194, the pUC19 origin of replication (ori pUC19), and the β-lactamase (ampicillin resistance) gene (bla).

Plasmid pSJ1130 (FIG. 10) was derived from pSJ1103 (FIG. 9) by deleting a 1.6 kb NsiI-PstI fragment, essentially resulting in a pUC19 plasmid containing a pUB110 plus-origin and a truncated rep gene (rep'). The plasmid was transformed into *E. coli* SJ6.

A 1.4 kb HindIII fragment from pDN3060 (FIG. 6) containing the plus-origin followed by the intact rep gene was then inserted into the unique HindIII site of pSJ1130 (FIG. 10), and the ligated plasmid was transformed into *E.coli* SJ6, resulting in pSJ1136 (FIG. 11). In this experiment, the fragment happened to be inserted into pSJ1136 in two tandem copies. One of these copies was subsequently deleted by digestion of pSJ1136 with NsiI, religation of the 5.1 kb fragment and transformation of *E. coli* SJ6, resulting in pSJ1137 (FIG. 12) which contains one pUB110 origin next to a truncated rep gene and one pUB110 origin next to an intact rep gene.

The gene encoding kanamycin resistance (kan) was excised from plasmid pPL1484 (FIG. 13) on a 1.4 kb SphI fragment and inserted in each of the two possible orientations into the SphI site of pSJ1137 (FIG. 12), followed by transformation of *E. coli* SJ6, resulting in pSJ1155 (FIG. 14) and pSJ1157 (FIG. 15), respectively. pSJ1157 contained the kan gene in two tandem copies. One copy was excised with BamHI, and the 6.5 kb fragment religated and transformed into *E. coli* SJ6 to form pSJ1259 (FIG. 16).

Plasmid pSJ1139(FIG. 18) was constructed in the following way: The Bacillus plasmid pDN2904 (FIG. 17), containing a chloramphenicol resistance gene (cat), a kanamycin resistance gene (kan) and the pUB110 plus-origin with the corresponding rep gene was digested with SphI and ligated to pSJ1130 (FIG. 10) which had also been digested with SphI. The resulting plasmid pSJ1139 contains a pUB110 origin associated with a truncated rep gene and a pUB110 origin associated with an intact red gene.

EXAMPLE 3

Formation of progeny DNA vectors from plasmids containing two pUB110 origins of replication in *Bacillus subtilis*

Plasmid pSJ1139 (FIG. 18) (prepared from *E. coli* SJ1139 in a manner known per se) was transformed into *B. subtilis* DN1885 selecting for kanamycin resistance (10 μg/ml), and plasmid DNA was prepared from several transformants. Agarose gel electrophoresis of these plasmids, whether undigested or digested with a variety of restriction enzymes, showed the presence of two smaller DNA molecules of 5.1 kb and 2.4 kb, respectively, as well as a small amount of the full-length plasmid pSJ1139 of 7.5 kb. The restriction patterns obtained were the expected ones for formation of the two progeny vectors pSJ1139a (FIG. 19) and pSJ1139b (FIG. 20), either by homologous crossing-over between the two rep-sequences on pSJ1139 or by the action of the Rep protein which produces a nick at the pUB110 plus-origin in the plus DNA strand which is then displaced and recircularized as described in A. Gruss and S. D. Erlich, op. cit. (both these mechanisms could lead to the same two progeny vectors.

These vectors were further analyzed by retransformation into *B. subtilis* strain DN1885, and plating on LB plates containing either 10 μg/ml kanamycin or 6 μg/ml chloramphenicol, followed by replica plating of each plate onto a new plate containing the other antibiotic. Vectors were then isolated from each type of transformant and analyzed by agarose gel electrophoresis with the following results:

Transformants resistant to both chloramphenicol and kanamycin contain all three vector species(pSJ1139 of 7.5 kb, pSJ1139a of 2.4 kb and pSJ1139b of 5.1 kb). Transformants resistant to chloramphenicol and sensitive to kanamycin only contain pSJ1139b. Transformants resistant to kanamycin but sensitive to chloramphenicol were not obtained. The small progeny vector pSJ1139a of 2.4 kb is thus not able to replicate autonomously in *B. subtilis*.

EXAMPLE 4

Stable integration of a non-replicative DNA molecule in the *B. subtilis* chromosome

Construction of a *B. subtilis* strain containing one chromosomal copy of a cyclodextrin glycosyl transferase (CGTase) gene

The CGTase gene (CGT) was excised from plasmid pPL1878 (FIG. 22) on a 2.5 kb BamHI-SphI fragment and ligated to the BamHI-SphI digested plasmid pDN3020 (FIG. 21) to form plasmid pPL1896 (FIG. 23). pDN3020 is a derivative of pDN1313 (Diderichsen, 1986), constructed by inserting a synthetic SphI-containing oligonucleotide linker (prepared as described in Example 1 above) into the EcoRI site of plasmid pDN1380 (Diderichsen and Christiansen, 1988) resulting in plasmid pDN1620. The promoter region from a maltogenic amylase from *B. stearothermophilus* (PamyM), present on pDN1620 (B. Diderichsen and L. Christiansen, op. cit.) was then transferred to SphI-BamHI digested pUC19 on an approximately 200 bp BamHI-SphI fragment resulting in plasmid pDN2977. The promoter region was excised from pDN2977 on an approximately 200

bp BglII-SacI fragment which was inserted into the polylinker region of pDN1313, thereby generating plasmid pDN3020. The CGTase gene on pPL1896 is flanked by two fragments of *B. subtilis* chromosomal DNA indicated as dal and dfs in FIG. 23. dal is the gene encoding D,L-alanine racemase of *B. subtilis* (Diderichsen 1986).

Plasmid pPL1896 was transformed into *B. subtilis* strain DN1686. When selecting solely for Dal$^+$ transformants, several strains were obtained that were chloramphenicol sensitive, CGTase$^+$. They were formed by a double homologous crossing-over between pPL1896 and the DN1686 chromosome, as described in Diderichsen, 1986. One such strain is PL1897, containing a chromosomally integrated copy of the CGTase gene.

Construction of an integration vector containing the CGTase gene

The CGTase gene was excised from pPL1878 (FIG. 22) on a 2.5 kb BamHI-NotI fragment. An expression vector was constructed by inserting a 0.6 kb SphI-PstI fragment containing the promoter region of the alpha-amylase gene cloned from an amylase-overproducing derivative of *B. licheniformis* ATCC9789 obtained by conventional mutagenesis procedures into a pUB110 derived vector containing the pUB110 origin and the gene encoding kanamycin resistance. The CGTase gene (cgt) was inserted downstream of this promoter between the BamHI and NotI sites, resulting in pSJ993 (FIG. 24).

A 4 kb BglII fragment from pSJ993 was inserted into the BglII site of pSJ1155 (described in Example 1 above, FIG. 14), the resulting plasmid was transformed into *E. coli* strain SJ6, and ampicillin-resistant, CGTase-producing transformants of *E. coli* SJ6 were isolated by plating transformants on LB plates containing 100 μg/ml ampicillin and 0.5% soluble starch, screening for the formation of a clear halo around the colonies after staining the plates with iodine vapour. A transformant harbouring the plasmid pSJ1163 (FIG. 25) in which the kanamycin resistance gene had been regenerated was kept for further experiments.

Formation of progeny vectors from pSJ1163 in *B. subtilis* strains DN1885 and PL1897

pSJ1163 (FIG. 25) was transformed into DN1885 and vector DNA was prepared from kanamycin-resistant transformants and analyzed by agarose gel electrophoresis. This showed traces of a 10.5 kb plasmid molecule, corresponding to pSJ1163, and two progeny vector molecules pSJ1163b of 4.1 kb (FIG. 27) and pSJ1163a of 6.4 kb (FIG. 26) in approximately equal and in far larger amounts, respectively, corresponding to progeny vectors derived either by homologous recombination between the two rep-sequences of pSJ1163 or by the action of the Rep protein at each plus origin in rolling circle replication as described above. The formation of progeny vectors as described above was also observed when pSJ1163 was transformed into PL1897, and two such transformants were kept for further experiments as strains SJ1168 and SJ1170.

Isolation of integrants containing non-replicative DNA molecules

Strains SJ1168 and SJ1170 were inoculated into 10 ml TY medium containing 5 μg/ml kanamycin and incubated overnight at 37° C. 100 μl of each culture were then inoculated into fresh TY medium and the incubation was repeated. After four such cycles of incubation overnight, plasmid DNA was prepared from the two cultures and analyzed by agarose gel electrophoresis. No plasmid molecules were observed. When the plasmid preparation was used to transform *E. coli* selecting for ampicillin resistance, no transformants were obtained, indicating that neither the original 10.5 kb pSJ1163 nor the 4.1 kb progeny vector molecule pSJ1163b were present. The kanamycin-resistant, plasmid-free strains were kept for further experiments as SJ1223 and SJ1237.

Amplification of integrated DNA

By selecting for growth in TY medium containing gradually increasing concentrations of kanamycin, strains were isolated that were able to grow in 20, 50, 100, 200, 400, 600, 800, 1000, 1200, and 1400 μg/ml kanamycin. In chromosomal DNA from strains resistant to above about 400 ug/ml kanamycin, digestions with NheI or NotI revealed a DNA band of the size expected from digestion of the 6.4 kb progeny vector pSJ1163a with these enzymes. This band did not appear in digests of DNA prepared from strains with a lesser degree of kanamycin resistance. A conservative estimate would be that at least 5–10 copies of the integrated DNA were present when this band appeared.

Stability of integrated DNA

Strains resistant to 400 μg/ml kanamycin were grown for one week at 37° C. in shake flasks containing BPX medium without any added kanamycin. They were then plated onto LB plates and subsequently replica plated onto plates containing 10 μg/ml kanamycin. Of about 100 colonies, all were kanamycin resistant, indicating the stable inheritance of the kanamycin resistance gene present on the integrated DNA in the abscence of selection pressure.

Stability of a plasmid-borne CGTase gene in *B. subtilis*

Plasmid pP11892 is essentially identical to pSJ993 (FIG. 24), the only difference being that a different polylinker region is present downstream of the CGTase gene. This plasmid was introduced into DN1885 and the resulting strain SJ984 was grown for one week at 37° C. in shake flasks containing BPX medium without any added kanamycin. Plating on kanamycin-containing plates (10 μg/ml) gave a 10-fold lower cell count than plates without kanamycin, indicating that 90% of the cells had lost their plasmid. This was also reflected by the finding that less than 10% of the colonies on plates without kanamycin produced CGTase.

EXAMPLE 5

Formation of progeny vectors from pSJ1156 in *B. licheniformis* ATCC 9789.

Plasmid pSJ1156 is identical to pSJ1157 shown in FIG. 15. pSJ1156 was introduced into *B. licheniformis* ATCC 9789 by protoplast transformation, selecting for kanamycin resistance, resulting in strain SJ1199. Analysis of the plasmid content of SJ1199 by restriction enzyme digestion and agarose gel electrophoresis revealed the presence of two plasmid molecules. One was identical to pSJ1259 (FIG. 16) and most likely formed by deletion of one copy of the kan gone by homologous recombination. The other corresponded to pSJ1259a (FIG. 28), one of the two progeny molecules that could be formed either by homologous recombination between the two rep sequences of pSJ1259 or by the action of the Rep protein at each plus origin in rolling circle replication as described above.

EXAMPLE 6

Stable integration of a non-replicative DNA molecule in the *B. licheniformis* ATCC 9789 chromosome

Construction of integration vector

Plasmid pSJ1260 is identical to pSJ1259 shown in FIG. 16. Chromosomal DNA from *B. licheniformis* ATCC 9789

was digested with PstI+BamHI and fragments between 2 and 4 kb were isolated from an agarose gel. These fragments were ligated into pSJ1260 digested with PstI+BamHI, and transformed into *E. coli* SJ6 selecting ampicillin resistance. One transformant obtained contained an insert of 2.1 kb and the plasmid was denoted pSJ1555 (FIG. 29). *E. coli* SJ6 containing pSJ1555 was deposited at the National Collection of Industrial and Marine Bacteria Ltd, 23 St. Machar Drive, Aberdeen, AB2 1RY, Scotland, UK on Dec. 12, 1990 in accordance with the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, with the accession number NCIMB 40346. This plasmid has the capacity to form the two progeny molecules pSJ1555a (FIG. 30) and pSJ1555b (FIG. 31).

Isolation of *B. licheniformis* integrant containing a non-replicative DNA molecule.

pSJ1555 was introduced into *B. licheniformis ATCC* 9789 by protoplast transformation, selecting for kanamycin resistance. One regenerated, kanamycin-resistant transformant (SJ1613) was plasmid-free as seen by gel electrophoresis of a plasmid-preparation from that transformant, and the plasmid preparation was unable to transform *B. subtilis* to kanamycin resistance. This result indicates that the non-replicative progeny molecule pSJ1555a had formed and had been integrated into the ATCC 9789 chromosome.

Amplification and stability of integrated DNA

Strain SJ1613 was grown in successive 10 ml TY cultures containing kanamycin at 10, 20, 50, 100, 200, 400, 600, 800, 1000, 1500, 2000, 2500, 3000, 4000 and 5000 ug/ml, and strains growing at each of these different concentrations were kept for further study. Strains resistant to 20, 200 and 1500 ug/ml kanamycin were further analyzed. Chromosomal DNA from the two latter strains revealed upon digestion with BamHI a distinct band of 4.5 kb, absent from DNA of the first strain, as expected for strains containing multiple copies of pSJ1555a integrated in the chromosome.

All strains were grown in BPX shake flasks at 37° C. for 7 days without kanamycin, and then streaked on LB plates. Replica plating from LB plates to kanamycin plates (10 ug/ml) revealed no kanamycin sensitive colonies. Colony counts on plates with and without kanamycin (10 ug/ml) were obtained for the three strains resistant to 20, 200 and 1500 ug/ml kanamycin, and were in all cases 10^{10} ml^{-1}, indicating stability of the integrated kan gene.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 3

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATTGATCAA GCTTTAAATG CATGCTAGCA ACGCGGCCGC CAACCTCGAG ATCTCATGCT 60

-continued

AGTTCGAAAT TTACGTACGA TCGTTGCGCC GGCGGTTGGA GCTCTAGAGT ACTTAA 116

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AATTCTGCAG ATATCAAGAT AAGAAAGAAC AAGTTCCG 38

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GATCCGGAAC TTGTTCTTTC TTATCTTGAT ATCTGCAG 38

We claim:

1. A method of producing a bacterial cell which in its genome carries an integrated non-replicative DNA construct comprising (1) a DNA sequence of interest, (2) a DNA sequence which is homologous with a region of the genome of the cell, and (3) an origin of replication, the DNA construct lacking a functional gene coding for a factor required to initiate replication from said origin of replication, the method comprising

(a) transforming bacterial cells with a parental plasmid vector which comprises a first origin of replication and a second origin of replication in the same orientation as the first origin of replication, which first and second origins of replication are sufficiently similar to be functional with the same replication factor,

the first and second origins of replication dividing the vector into two parts, (i) a first part comprising the first origin of replication and a gene encoding a replication factor required for plasmid replication from said first and second origin of replication, and (ii) a second part comprising the second origin of replication, a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the vector, and

(b) culturing the transformed cells under selective conditions, replication of the parental plasmid vector giving rise to the formation of a first progeny vector comprising the first origin of replication and a functional gene encoding a replication factor required for plasmid replication from said first and second origin of replication, and a second progeny vector comprising the second origin of replication but lacking a functional gene encoding a replication factor, as well as comprising a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of the cell, continued culturing of the transformed cells under selective conditions resulting in the integration of said second progeny vector into the bacterial genome by homologous recombination and loss of the first progeny vector as well as the parental vector from the cells.

2. A method according to claim 1, wherein the second origin of replication is derived from the same plasmid as the first origin of replication.

3. A method according to claim 1, wherein a gene encoding the replication factor associated with the second origin of replication of the second progeny vector of step(b) has been deleted.

4. A method according to claim 1, wherein a gene encoding the replication factor associated with the second origin of replication of the second progeny vector of step(b) has been modified.

5. A method according to claim 4, wherein the gene encoding the replication factor has been modified by deletion, insertion or substitution of one or more nucleotides of the DNA sequence of the gene, or by deletion of transcriptional or translational start or stop signals.

6. A method according to claim 1, wherein the parental plasmid vector is one which is unable to replicate at increased temperatures which yet permit growth of the host cell, and wherein the bacterial cells are initially cultured at a temperature permitting plasmid replication and subsequently, after integration of the second progeny vector into the bacterial genome, cultured at a temperature which does not permit plasmid replication so that the first progeny vector as well as the parental vector are lost from the cells.

7. A method according to claim 1 in which the first and second origins of replication associated with the parental vector are each derived from a single-stranded DNA plasmid.

8. A method according to claim 7, wherein the second origin of replication is derived from the same single-strand DNA plasmid as the first origin of replication.

9. A method according to claim 1, wherein the bacterial cell is a cell of a gram-positive bacterium.

10. A method according to claim 9, wherein the gram-positive bacterium is a strain belonging to the genus Bacillus or Streptomyces.

11. A method according to claim 10, wherein the bacterium is a strain of *Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus subtilis* or *Streptomyces lividans*.

12. A method of producing a bacterial cell which in its genome carries an integrated non-replicative DNA construct comprising (1) a DNA sequence of interest, (2) a DNA sequence which is homologous with a region of the genome of the cell, and (3) an origin of replication, the DNA construct lacking a functional gene coding for a factor required to initiate replication from said origin of replication, the method comprising

(a) transforming bacterial cells with (i) a first DNA vector comprising a first origin of replication and a functional gene encoding a factor required for plasmid replication from said first origin of replication, and with (ii) a second DNA vector comprising a second origin of replication but lacking a functional gene encoding a factor required for plasmid replication from the second origin of replication, as well as comprising a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of the cell,

said first and second origins of replication being sufficiently similar to be functional with the same replication factor so that replication of the second DNA vector from the second origin of replication is initiated by the replication factor encoded by the gene present on the first DNA vector, and

(b) culturing the resulting cells under selective conditions leading to integration of said second DNA vector into the bacterial genome by homologous recombination and loss of the first DNA vector.

13. A method according to claim 12, wherein the second origin of replication is derived from the same plasmid as the first origin of replication.

14. A method according to claim 12, wherein the second DNA vector has been deleted of the gene encoding the replication factor associated with the second origin of replication.

15. A method according to claim 14, wherein the gene encoding the replication factor associated with the second origin of replication has been modified by deletion, insertion or substitution of one or more nucleotides of the DNA sequence of the gene, or by deletion of transcriptional or translational start or stop signals.

16. A method according to claim 12, wherein the second DNA vector further comprises a selectable marker.

17. A method according to claim 12, wherein the first DNA vector comprises a first plus origin of replication from a single-strand DNA plasmid and a functional rep gene, and wherein the second DNA vector comprises a second plus origin of replication from a single-strand DNA plasmid but lacking a functional rep gene cognate to the second plus origin of replication, as well as comprising a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of said cell.

18. A method according to claim 17, wherein the second plus origin of replication is derived from the same single-strand DNA plasmid as the first plus origin of replication.

19. A method according to claim 12, wherein the first DNA vector is one which is unable to replicate at increased temperatures which yet permit growth of the host cells, and wherein the bacterial cells are initially cultured at a temperature permitting plasmid replication and subsequently, after integration of the second DNA vector into the bacterial genome, cultured at a temperature which does not permit plasmid replication so that the first DNA vector is lost from the cells.

20. A method according to claim 12, which is a cell of a gram-positive bacterium.

21. A method according to claim 20, wherein the gram-positive bacterium is a strain belonging to the genus Bacillus or Streptomyces.

22. A method according to claim 21, wherein the bacterium is a strain of *Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus subtilis* or *Streptomyces lividans*.

23. A bacterial cell obtainable according to the method of claim 1 or 12 which in its genome carries an integrated non-replicative DNA construct comprising (1) a DNA sequence of interest, (2) a DNA sequence which is homologous with a region of the genome of the cell, and (3) an origin of replication, wherein the DNA construct has been deleted of a gene coding for a factor required to initiate replication from said origin of replication or wherein the gene encoding the replication factor has been modified so as to encode an inactive replication factor.

24. A cell according to claim 23, wherein the DNA construct has been deleted of the gene encoding the replication factor.

25. A cell according to claim 23, wherein the gene encoding the replication factor has been modified so as to encode an inactive replication factor.

26. A cell according to claim 25, wherein said gene has been modified by deletion, insertion or substitution of one or more nucleotides of the DNA sequence of the gene, or by deletion of transcriptional or translational start or stop signals.

27. A cell according to claim 23, wherein the DNA construct comprises a DNA sequence of interest, a DNA sequence which is homologous to a region of the genome of the cell, and a plus origin of replication from a single-strand DNA plasmid, the DNA construct lacking a functional rep gene cognate to the plus origin of replication.

28. A cell according to claim 23, wherein the DNA construct additionally comprises a selectable marker.

29. A cell according to claim 23, which is a cell of a gram-positive bacterium.

30. A cell according to claim 29, wherein the gram-positive bacterium is a strain belonging to the genus Bacillus or Streptomyces.

31. A cell according to claim 30, wherein the bacterium is a strain of *Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus subtilis* or *Streptomyces lividans*.

32. A process for producing a polypeptide of interest, comprising culturing a bacterial cell according to claim 23 containing an integrated DNA sequence which codes for said polypeptide under conditions conducive to the production of the polypeptide and recovering the resulting polypeptide from the culture.

33. A process according to claim 32, wherein the polypeptide is an enzyme.

34. A process according to claim 33, wherein the enzyme is a protease, amylase or lipase.

35. A parental plasmid vector which comprises a first origin of replication and a second origin of replication in the same orientation as the first origin of replication, which first and second origins of replication are sufficiently similar to be functional with the same replication factor(s),

the first and second origins of replication dividing the vector into two parts, (i) a first part comprising the first origin of replication and one or more functional genes

encoding the replication factor(s) required for plasmid replication from said first and second origin of replication, and (ii) a second part comprising the second origin of replication, a DNA sequence of interest, and a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the vector.

36. A plasmid vector according to claim **35**, wherein the second origin of replication is derived from the same plasmid as the first origin of replication.

37. A plasmid vector according to claim **35**, which has been deleted of the gene encoding the replication factor associated with the second origin of replication.

38. A plasmid vector according to claim **35**, wherein the gene encoding the replication factor associated with the second origin of replication has been modified.

39. A plasmid vector according to claim **38**, wherein the gene has been modified by deletion, insertion or substitution of one or more nucleotides of the DNA sequence of the gene, or by deletion of transcriptional or translational start or stop signals.

40. A plasmid vector according to claim **35** in which the first and second origins of replication associated with the parental vector are each derived from a single-stranded DNA plasmid.

41. A plasmid vector according to claim **40**, wherein the second origin of replication is derived from the same single-strand DNA plasmid as the first origin of replication.

42. A plasmid vector according to claim **35**, which further comprises a selectable marker.

43. A recombinant DNA vector comprising (1) a DNA sequence of interest, (2) a DNA sequence which is homologous with a region of the genome of the cell, and (3) an origin of replication, the DNA construct lacking a functional gene coding for a factor required to initiate replication from said origin of replication.

44. A vector according to claim **43**, which has been deleted of the gene encoding the replication factor.

45. A vector according to claim **43**, wherein the gene encoding the replication factor has been modified so as to encode an inactive replication factor.

46. A vector according to claim **45**, wherein said gene has been modified by deletion, insertion or substitution of one or more nucleotides of the DNA sequence of the gene, or by deletion of transcriptional or translational start or stop signals.

47. A vector according to claim **43**, comprising a DNA sequence of interest, a DNA sequence which is homologous with a region of the genome of a cell intended for introduction of the vector, and a plus origin of replication from a single-strand DNA plasmid, the vector lacking a functional rep gene associated with the plus origin.

48. A vector according to claim **43**, which additionally comprises a selectable marker.

49. A bacterial cell which comprises a first DNA vector comprising an origin of replication and one or more functional genes encoding the factor(s) required for plasmid replication from said first origin of replication, and a second DNA vector according to claim **43**.

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REVIEW ARTICLE

Molecular biology of the *Borrelia*, bacteria with linear replicons

I. Saint Girons,[1] I. G. Old[1] and B. E. Davidson[2]

Author for correspondence: I. Saint Girons. Tel: +33 1 45 68 83 66. Fax: +33 1 40 61 30 01. e-mail: isgirons@pasteur.fr

[1]Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, 75724 Paris cedex 15, France
[2]Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia

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Borrelia and Lyme disease

The genus *Borrelia* was known more than a century ago following the discovery of *Borrelia recurrentis* as the causative agent of epidemic relapsing fever. Interest in *Borrelia* increased dramatically after the recent discovery of Lyme disease and the observation that it is caused by a novel member of the genus. *Borrelia* are spirochaetes, organisms that comprise a separate phylum in the kingdom of eubacteria (Woese, 1987). They differ from other bacteria in having a helical shape with multiple waves and endoflagella which are located between the inner and outer membrane. In this review, we concentrate on the molecular biology of the causative agents of Lyme disease and North American relapsing fever (*Borrelia burgdorferi sensu lato* and *Borrelia hermsii*, respectively) because they have received the most research attention. To contain the review within reasonable limits we have not attempted to cover the taxonomy of the *Borrelia* or the widespread literature on antigenic variation of *B. hermsii*. Interested readers are referred to recent reviews on these topics by Barbour (1990) and Wilske *et al.* (1992).

Lyme disease is a multisystemic disorder which involves the joints, heart and nervous system (for a review see Steere, 1989). The disease was shown to be transmitted by ticks of the genus *Ixodes* and caused by a spirochaete (Burgdorfer *et al.*, 1982). Analysis of these spirochaetes revealed that they were from a new species of *Borrelia* (Hyde & Johnson, 1984), which was named *B. burgdorferi* in honour of its discoverer, W. Burgdorfer (Johnson *et al.*, 1984). The delineation of a single species was based on DNA/DNA hybridization studies performed on a small number of strains that originated from the USA (Hyde & Johnson, 1984). Subsequent DNA/DNA hybridization, 16S rRNA sequencing and ribotyping of strains from different geographical origins in Europe and the USA led to the realization that at least three different species are responsible for Lyme disease: *B. burgdorferi sensu stricto*, *B. garinii* and *B. afzelii* (Postic *et al.*, 1990; Baranton *et al.*, 1992; Marconi & Garon, 1992; Canica *et al.*, 1993). The existence of several different aetiological agents for Lyme disease is correlated with different patterns of symptoms seen in Europe and the USA (Assous *et al.*, 1993; Van Dam *et al.*, 1993). We use the term *B. burgdorferi* to refer to *B. burgdorferi sensu stricto* and Lyme disease spirochaetes to cover *B. burgdorferi sensu stricto*, *B. garinii* and *B. afzelii* (formerly group VS461). A fourth species, *B. japonica*, was recently isolated from Japanese ticks (Kawabata *et al.*, 1993; Postic *et al.*, 1993).

Initially, studies on *Borrelia* spp. proceeded slowly, due to the lack of a suitable medium for cultivating the organism. Even now, *Borrelia* spp. such as *B. recurrentis* cannot be cultivated *in vitro*. The liquid medium defined by Kelly for the growth of *B. hermsii* and found suitable for the growth of *B. burgdorferi* was improved by Barbour, Stoenner and Kelly to give BSKII medium, which is now used universally to culture Lyme disease *Borrelia* and *B. hermsii* (Barbour, 1984). In particular, BSKII medium contains *N*-acetylglucosamine, an essential component of the chitin in arthropod cuticles. Some other components, for example gelatin, are not essential for growth but their presence improves the cell yield to modest but usable levels (up to 2×10^8 bacteria per ml). The doubling time of *B. burgdorferi* is between 6 and 12 h (Barbour & Hayes, 1986). Colonies were obtained for the first time on solid BSKII medium a few years ago (Kurtii *et al.*, 1987).

Investigations of the molecular biology of *Borrelia* spp. have been hampered by the unavailability of tools for genetic transfer. Consequently, both classical and reverse genetics have not been possible. Methods enabling the genetic manipulation of spirochaetes have so far remained elusive. Indeed, the only published report describes electroporation of *Serpulina hyodysenteriae* (Ter Huurne *et al.*, 1992).

The GenBank accession number for the sequence reported in this paper is U04527 (gb-ba: BBU04527).

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The *Borrelia* genome

Lyme disease spirochaetal DNA has a low G+C content of 28·0–30·5 mol% (Johnson *et al.*, 1984; Baranton *et al.*, 1992). This value is comparable with that found in other members of the genus and is significantly less than that of other spirochaetes such as *Treponema denticola* (36·0 mol%), *Leptospira interrogans sensu lato* (35–40 mol%) (Haapala, 1969; Hyde & Johnson, 1984) and *Treponema pallidum* (53 mol%) (Miao & Fieldsteel, 1980).

As with many bacteria, the genome of *B. burgdorferi* consists of a chromosome and a number of different plasmids. The feature that distinguishes the borrelial genome from most other bacterial genomes is that the chromosome and some of the plasmids are linear DNA molecules (Fig. 1). This unusual property has enhanced interest in the molecular genetics of *B. burgdorferi* and related species. Up to now, linear chromosomes have also been described for *Streptomyces lividans* (Lin *et al.*, 1993) and *Agrobacterium tumefaciens* (Allardet-Servent *et al.*, 1993), while linear plasmids have been observed in *Streptomyces* spp. (Kinashi *et al.*, 1987).

The linear chromosome of Lyme disease spirochaetes

Discovery of the linearity by pulsed field gel electrophoresis (PFGE). The discovery that the chromosome is linear was made concurrently in two laboratories when high-molecular-mass *B. burgdorferi* DNA was subjected to analysis by PFGE (Baril *et al.*, 1989; Ferdows & Barbour, 1989). The prior discovery of linear plasmids in this organism was helpful in interpreting these analyses (Barbour & Garon, 1987). The DNA for the PFGE experiments was prepared *in situ* in agarose blocks by procedures that have been established with other organisms to minimize shearing. Large open circular DNA molecules such as bacterial chromosomes do not migrate from the origin during PFGE, presumably because the looped molecules become impaled on spurs of agarose (Serwer & Hayes, 1987). Surprisingly, the majority of the *B. burgdorferi* chromosomal DNA was not retained at the origin but migrated as a band with mobility approximately equal to that of a linear duplex molecule of 1 Mbp. Digestion of the DNA with different endonucleases before electrophoresis yielded in each case a number of fragments of total molecular mass between 900–1000 kbp. The possibility that the 1 Mbp DNA was composed of supercoiled circular molecules was eliminated because its electrophoretic mobility was unaffected by the introduction of supercoil-relaxing single-strand breaks. The conclusion from these experiments was that the *B. burgdorferi* chromosome is a linear molecule of between 900 and 1000 kbp.

Chromosome copy number has not yet been reported for a Lyme disease spirochaete, but a detailed investigation with *B. hermsii*, which also has a 1 Mbp linear chromosome, revealed a relatively high value of 16 copies of chromosome per cell for *B. hermsii* grown in mice (Kitten & Barbour, 1992). By contrast, actively growing *Escherichia coli* has on average 6·5 chromosomal origins and 1·9 termini per cell, while *Azotobacter vinelandii* contains up to 40 chromosomal copies per cell (Bremer & Dennis, 1987; Punita *et al.*, 1989).

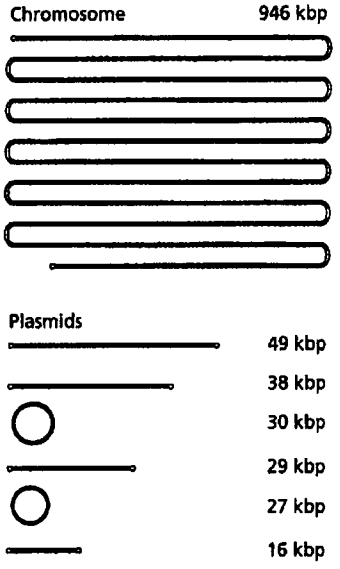

Fig. 1. Schematic scale representation of the genome of *B. burgdorferi* B31 (the type strain). The depiction of the ends of linear replicons as single-strand loops is speculative, except for the 16 kbp plasmid (Hinnebusch & Barbour, 1991). The complement of replicons shown is that reported by Sadziene *et al.* (1993b).

Physical and genetic maps of the linear chromosome. A physical map of the chromosome of *B. burgdorferi* was first reported for strain 212 (Davidson *et al.*, 1992). In determining the map extensive use was made of digestions in agarose blocks of chromosomal DNA or restriction fragments that had been purified by PFGE. The use of chromosomal DNA, free of the linear plasmids, eliminated uncertainties in the analysis of the digestion products. A total of 47 digestion sites were located for the enzymes *Sgr*AI, *Sac*II, *Mlu*I, *Bss*HII, *Eag*I, *Sma*I, *Nae*I and *Apa*I. In most regions of the map the gap between restriction sites was 50 kbp or less, enabling good resolution for the mapping of genetic loci. A number of important conclusions were reached from the mapping studies. First, by summing the sizes of the different digestion products an accurate value of 946 kbp was obtained for the size of the *B. burgdorferi* chromosome. This value agreed well with that reported in the initial PFGE studies described above. Second, the data obtained in the mapping studies were consistent with the chromosome being linear. The alternative explanation that the chromosome is circular would require the presence of a $< 1{\cdot}5$ kbp region of the chromosome which contained at least one site each for *Sgr*AI, *Sac*II, *Bss*HII, *Mlu*I and *Sma*I. This explanation is unlikely given the low G+C content of the *B. burgdorferi*

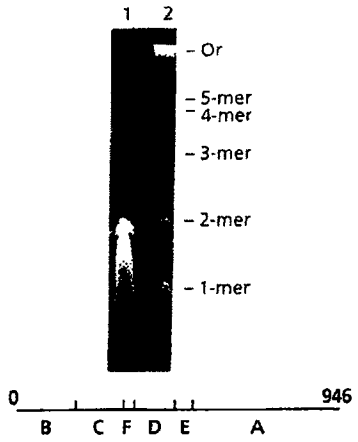

Fig. 2. PFGE separation of self-ligated *MluI* fragments of the *B. burgdorferi* 212 chromosome. Fragments MlB and MlC were purified by PFGE, and the agarose blocks containing them were excised, washed and then incubated with DNA ligase for 36 h at 10 °C. The blocks were embedded in a second agarose gel and the ligation products were resolved by further PFGE. Lanes: 1, MlB; 2, MlC. The *MluI* map of *B. burgdorferi* 212 is shown at the bottom of the figure (Davidson *et al.*, 1992).

chromosome. One possibility that cannot be eliminated by any of the experiments that have used PFGE is that the *B. burgdorferi* chromosome contains an extremely fragile locus which is broken during the preparation of high-molecular-mass DNA. It may require the determination of the structure of the chromosomal extremities to be able to exclude this possibility unequivocally.

The molecular structures of the chromosomal telomeres are currently unknown, and constitute an important area of investigation. The following experiment provides some relevant information. *Mlu*I fragments of the *B. burgdorferi* 212 chromosome, purified by PFGE electrophoresis, were self-ligated *in situ* in the agarose and then resubjected to PFGE (Fig. 2). The internal fragment, MlC, yielded a ladder of bands corresponding to monomer, dimer, trimer, tetramer and pentamer as well as a significant quantity of presumably open circular polymeric DNA which remained in the well. By contrast, the end fragment, MlB, yielded only the monomeric and dimeric species, indicating that the telomeric ends were not ligatable. Attempted self-ligation of undigested chromosomal DNA gave no higher polymers (data not shown), again indicating non-ligatable telomeres.

Determination of the restriction map of a second *B. burgdorferi* strain (Sh-2-82, isolated in New York) enabled interesting conclusions to be reached about the stability of the chromosomes within the species (Casjens & Huang, 1993). The size of the Sh-2-82 chromosome, 952 ± 12 kbp, was not significantly different from that of the 212 chromosome, but 8 of the 41 sites for the restriction enzymes *Sgr*AI, *Sac*II, *Mlu*I, *Bss*HII, *Eag*I and *Sma*I were

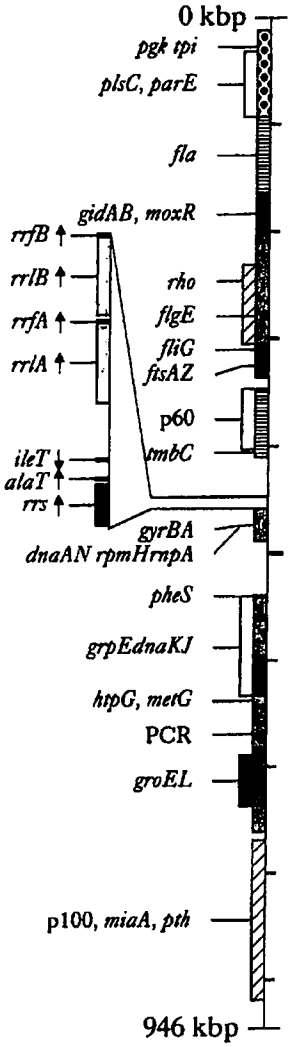

Fig. 3. Genetic map of the *B. burgdorferi* 212 chromosome. Details of the individual genes can be obtained from the GenBank or EMBL databases, with the exception of *pheST* (A. Barbour, unpublished), *fliG* and *flgE* (N. Charon, unpublished), *ftsA, gidB, htpG, metG, miaA, moxR, parE, pgk, plsC, pth, tmbC* and *tpi* (I. G. Old, unpublished).

in dissimilar locations. This means that the nucleotide sequences of the chromosomes of the two strains differ by at least 3·2 % in the 248 bp that make up the relevant sites.

The unavailability of gene transfer systems in Lyme disease spirochaetes has prevented the use of classical genetic procedures to construct a genetic map for this organism. The availability of a physical map of the *B. burgdorferi* chromosome made it possible to commence genetic mapping of the chromosome by other means (Davidson *et al.*, 1992; Old *et al.*, 1992b; Casjens & Huang, 1993). The procedures used were simple: Southern blots of PFGE separations of restriction digests

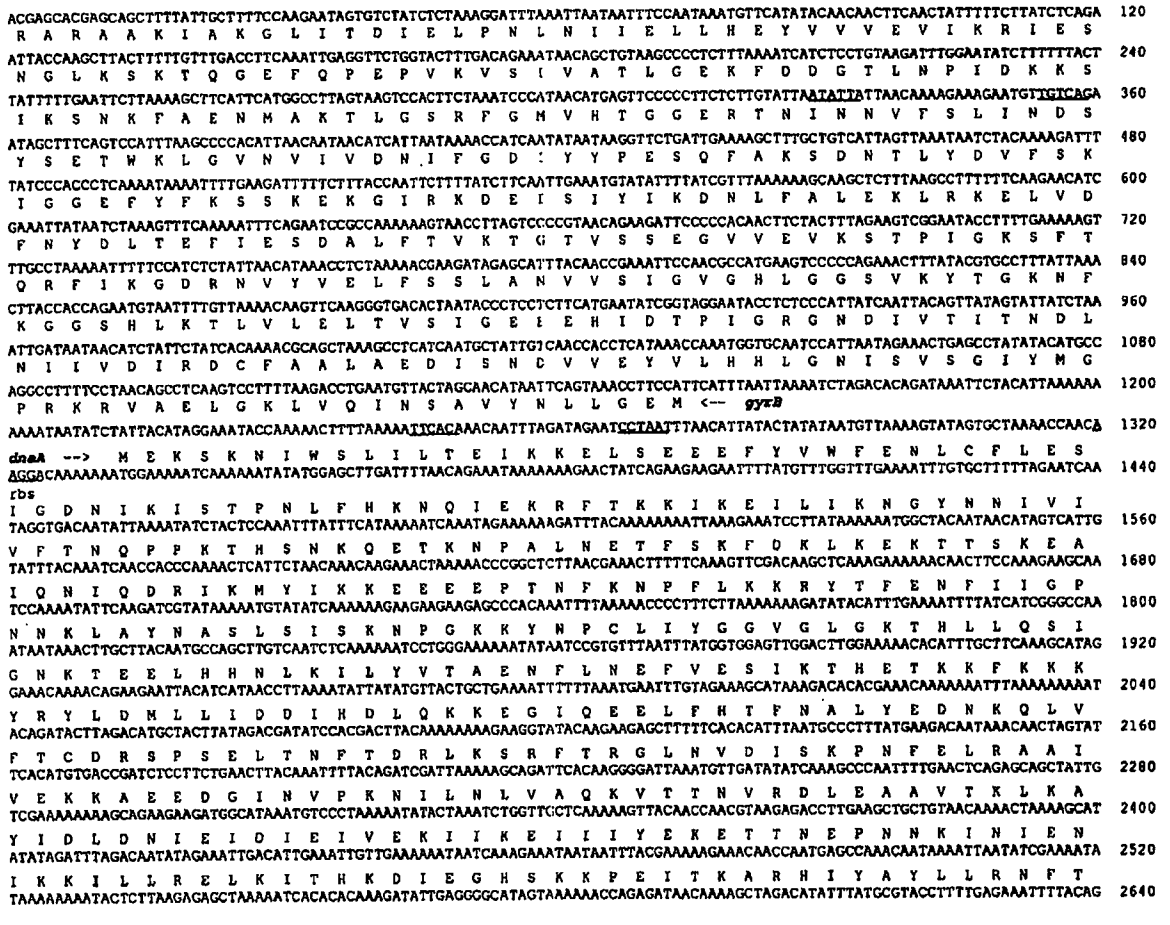

Fig. 4. For legend see facing page.

were hybridized with probes of characterized genes, thereby revealing the identity of the fragments carrying the genes. The resolution of the physical map determines the resolution that can be obtained by this approach. Since certain areas of the chromosome are richer than others in sites for the endonucleases used for mapping, there is considerable variation in the degree of precision with which different genes can be mapped by this process. Thus the chromosomal locations of genes such as *gidA* and *fla* were determined only approximately, whereas it was possible to map the locations of the rRNA genes and *pheS* precisely because they contained sites for one of the endonucleases used. The rDNA cluster, *fla*, *flgE*, *rho*, and the *dnaA gyrBA* cluster, *pheS*, the *grpE dnaKJ* cluster, and *groEL* have been located on both the *B. burgdorferi* 212 and Sh-2-82 maps and all loci mapped in identical positions of the genome, within the limits of precision of the mapping procedure. Our current map of the *B. burgdorferi* 212 chromosome has a total of 39 genetic loci (Fig. 3).

Few comparisons of chromosomal gene organization within a bacterial species have been performed. In six strains of *Mycoplasma mycoides* subsp. *mycoides* the relative positions of nine loci were found to be conserved while one gene had undergone a translocation (Pyle *et al.*, 1990). Analysis of different strains of *Clostridium perfringens* belonging to different biotypes showed that the overall organization of the chromosome in that bacterium is well-conserved (Canard *et al.*, 1992). By contrast, the chromosomes of two strains of *L. interrogans* exhibit several major DNA rearrangements (Zuerner *et al.*, 1993) and detailed comparison of six commonly used *E. coli* K12 strains revealed insertions and deletions ranging in size from 1 to 86 kbp (Perkins *et al.*, 1993).

We have recently extended our mapping studies to include the chromosomes of *B. garinii* and *B. afzelii* (C. Ojaimi, B. Davidson, I. Saint Girons & I. Old, unpublished). Interestingly, all of the 30 genetic loci examined have been found to have the same relative positions in the *B. burgdorferi*, *B. garinii* and *B. afzelii* chromosomes. Since these loci are dispersed throughout the entire chromosome this observation indicates the absence of major chromosomal translocation events during speciation and

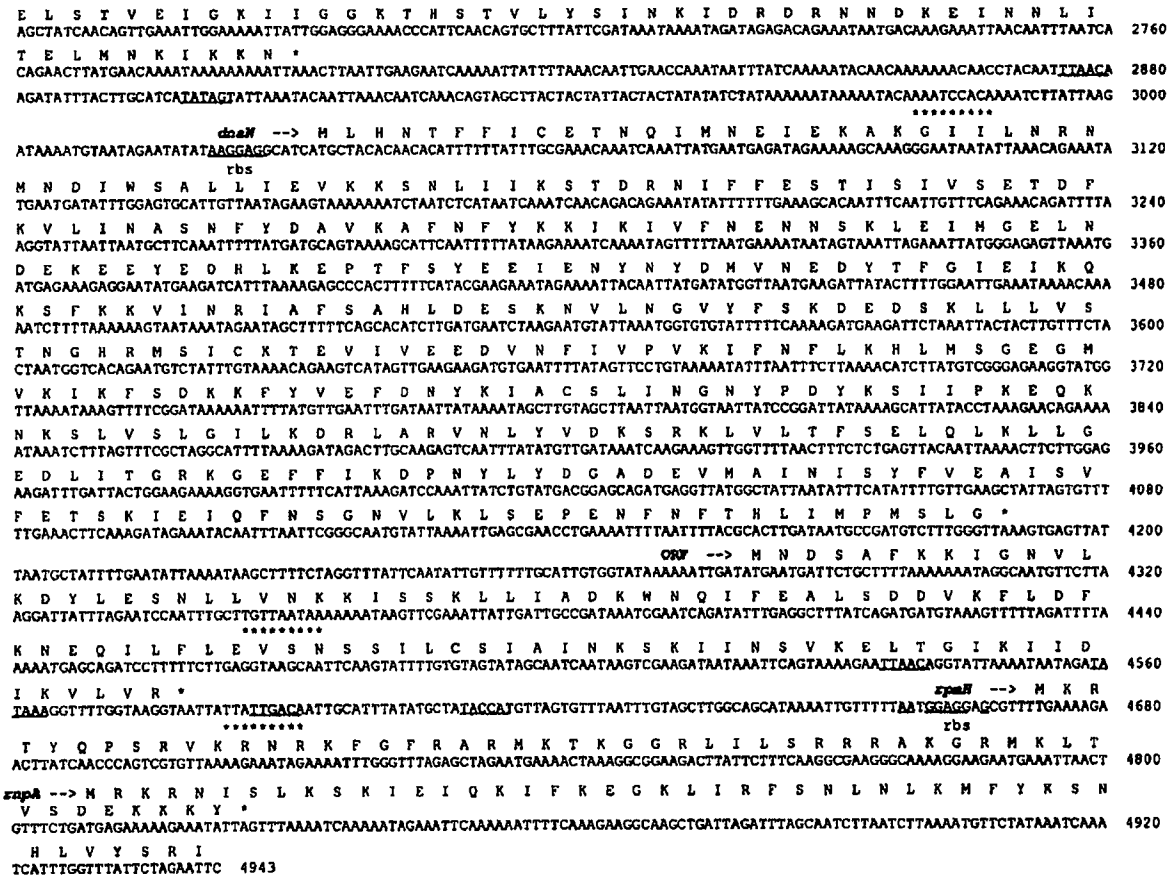

Fig. 4. Nucleotide sequence of 4943 bp in the region of the *B. burgdorferi gyrBA, dnaAN, rpmH, rnpA* gene cluster (Old *et al.*, 1992a, b, 1993a, b). The noteworthy factors are the inversion of *dnaA* and *dnaN* (see Fig. 5) and the absence of *recF* in the region, compared to the organization in *E. coli*, *B. subtilis* and *P. putida*. The coding strand is presented (except for *gyrB*). Possible 'Pribnow boxes' of promoters are underlined, while potential ribosome-binding sites with identity with the 3' end of the *B. burgdorferi* 16S RNA (5'-CACCUCCUUU-3') are underlined and marked 'rbs'. Putative DnaA boxes are marked by '*********'. The primary structure of the corresponding polypeptides, as deduced from the nucleotide sequence, is indicated in the one letter code. This sequence, which has been assigned the GenBank accession number U04527, includes the sequences previously designated L16681, L14948, L04647 and Z12166.

a significant degree of chromosomal stability. Whether stability of this type has also occurred at the micro level will not be known until high resolution maps, possibly generated by DNA sequencing, are available for comparison.

The origin of replication – a novel arrangement? The circular chromosomes found in bacteria such as *E. coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* have specific regions where replication of the chromosome is initiated (for a review see Smith *et al.*, 1991). These origins of replication (*ori*) contain at least four characteristically arranged 9 bp nucleotide repeats (DnaA boxes) which are sites for the binding of the protein DnaA, the initial step in replication (Holz *et al.*, 1992). Subsequently, DnaB and DnaC bind to the DnaA–DNA complex, replication commences and proceeds bidirectionally around the chromosome. Termination of replication also takes place at a specific site on the chromosome, *ter*, which is located opposite *ori* on the chromosomal map. It is of some interest to determine how this process has been adapted to achieve the replication of the linear borrelial chromosomes.

One strategy used was to isolate *B. burgdorferi* homologues of genes that have a chromosomal location near to *ori* in better studied bacteria (Old *et al.*, 1992a, b, 1993a, b). The identification of genes under these circumstances rested upon sequence homology, not functional properties. Use of this approach led to the discovery of a gene cluster in *B. burgdorferi* which contains the homologues of *rnpA*, *rpmH*, *dnaN*, *dnaA* and *gyrBA* in that order (Fig. 4). This gene order differs from the normally highly conserved

Fig. 5. Genetic organization in the *dnaA* regions of three typical (*Bacillus subtilis*, *P. putida* and *E. coli*) and three atypical eubacteria (*Buchnera aphidicola*, *Mycoplasma capricolum* and *Borrelia burgdorferi*). The figure is aligned with respect to the *dnaA* gene. Open reading frames (boxed regions) and intergenic regions (gaps) are drawn approximately to scale. The direction of transcription is shown by single-headed horizontal arrows while origin regions are marked with vertical arrows (in *Bacillus subtilis* a fourth origin is located between *gyrA* and *rrnO*). The genes shown are: *gidA*, *gidB* (glucose inhibited division proteins); 50K, 60K (proteins of undetermined function); *rnpA* (ribonuclease P, protein component); *rpmH* (ribosomal protein L34); *dnaA* (chromosome replication initiation protein); *dnaN* (DNA polymerase III, β-subunit); *recF* (inducer of SOS DNA repair); *gyrB*, *gyrA* (DNA gyrase, B- and A-subunits); *ksgA*, [*S*-adenosyl methionine-6-*N'*,*N'*-adenosyl (rRNA) dimethyltransferase]; *licA*, (outer membrane oligosaccharide biosynthesis); *orf* (open reading frames). For *Borrelia burgdorferi* 212 the direction of transcription of *gidAB* relative to *dnaA* is not known, and its placement on the downstream side of *gyrA* is inferred from the genetic arrangement in *B. afzelii*.

regions found in other eubacteria in a manner suggestive of a rearrangement during the evolution of the *B. burgdorferi* chromosome (Fig. 5). The *dnaA* gene cluster was found to be located almost precisely in the centre of the *B. burgdorferi* chromosome, close to the rDNA genes (Old *et al.*, 1992b; Casjens & Huang, 1993).

While the *dnaA* region of eubacteria is normally highly conserved, the number of origins of replication is not, varying from one for *E. coli* to four for *Bacillus subtilis* (Fig. 5). In *E. coli*, *P. putida* and *B. subtilis*, characteristic groups of DnaA boxes are located either adjacent to or near the gene *gidA*. In *P. putida* and *B. subtilis*, a second origin is located between *dnaA* and *rpmH*, while in *B. subtilis* there is a third origin between *dnaA* and *dnaN* and a fourth between *gyrA* and *rrnO*.

The *dnaA* region is considered to contain the ancestral origin of replication. A search for DnaA boxes (5'-TTATCCACA-3') in the *B. burgdorferi dnaA* region revealed none in the intergenic DNA region surrounding *dnaA* and two upstream of *rpmH* (Old *et al.*, 1993a). In *E. coli*, *oriC* is located upstream of *gidA*, approximately 40 kbp from *dnaA* (Fig. 5), while there is a single DnaA box in the *dnaA* regulatory region, essential for autoregulation of the gene. The *B. burgdorferi gidA* homologue has been cloned and mapped (Old *et al.*, 1992b). As in *E. coli*, the *B. burgdorferi* gene has been translocated relative to *dnaA* and *gidA* lies over 250 kbp from the central *dnaA* cluster. Determination of the nucleotide sequence around *gidA* should provide useful information on this point.

It is possible that the nucleotide sequences and/or arrangement of *B. burgdorferi* DnaA boxes are atypical. In this respect, it is interesting to note that *B. burgdorferi* DnaA differs in the normally highly conserved region which may be involved in DNA binding (Old *et al.*, 1993a).

Thus at this stage, the available data on the location of *ori* in the *B. burgdorferi* chromosome are inconclusive. A central location near *dnaA* has the appeal of symmetry, since bidirectional replication would proceed divergently to each telomere, where termination should occur more or less concurrently. As has been suggested previously (Old *et al.*, 1992b), this arrangement could have evolved from a circular chromosome by deletion of the DNA opposite the origin which normally encodes information for replication termination. Termination at the telomeres of the linear chromosome would not require this information. If *ori* is located near *gidA*, a different pathway for the evolution of the linear chromosome must have been

followed, e.g. deletion of the terminus region followed by translocation of *gidA*.

An unusual organization of rRNA genes and ribosomal protein operons. The arrangement and organization of the rRNA genes in the Lyme disease spirochaetes is unusual among bacteria in that there are two copies each of *rrl* (23S) and *rrf* (5S) but only one copy of *rrs* (16S) (Davidson *et al.*, 1992; Fukunaga *et al.*, 1992; Schwartz *et al.*, 1992). *rrl* and *rrf* are tandemly duplicated in the order *rrlA rrfA rrlB rrfB*, while *rrs* is located more than 2 kbp upstream of *rrlA*. Genes encoding $tRNA^{Ala}$ and $tRNA^{Ile}$ are present in this intervening DNA (Schwartz *et al.*, 1992; Gazumyan *et al.*, 1994). Normally, rRNA genes are present in operons that have the order: promoter, *rrs*, *rrl*, *rrf*. This arrangement results in the synthesis of equimolar amounts of the three rRNA species. The pattern of transcription of the rRNA genes in these *Borrelia* species has not been reported, so the manner in which it is regulated (presumably to achieve equimolar amounts of the three rRNA species) is not clear. It is likely that the genes of each *rrl rrf* pair are cotranscribed in a single transcript, since there is little DNA between them which could specify a promoter (Schwartz *et al.*, 1992). On the other hand, the presence of a relatively good *E. coli*-like promoter sequence in the 182 bp spacer between *rrfA* and *rrlB* raises the possibility of independent transcription of each *rrl rrf* pair (Schwartz *et al.*, 1992). This unusual rRNA gene arrangement is typical of Lyme disease spirochaetes since *B. anserina*, *B. turicatae* and *B. hermsii* each have only a single copy of both *rrl* and *rrf* (Schwartz *et al.*, 1992). Thus, the duplication of *rrl* and *rrf* has evolved recently in the Lyme disease spirochaetes. The nature of the mechanism whereby co-ordinate synthesis of the 16S and oither rRNAs is achieved, assuming that one exists, is an interesting topic for further investigation.

Recent work by Schwartz's group indicates that in *B. burgdorferi* the gene encoding elongation factor Tu (*tuf*) is not part of the *str* operon, which usually comprises the genes encoding S12 and S7 ribosomal proteins and elongation factors EfG and EfTu in other bacteria. Instead, it is located only 50 bp upstream of *rpsJ*, which encodes the ribosomal protein S10 (Gazumyan *et al.*, 1994). The gene order of the operon which encoes the S10 ribosomal protein matches that found in *E. coli*. The implications of this unusual arrangement for regulation of ribosomal protein and elongation factor Tu synthesis in *B. burgdorferi* are being determined.

Linear and circular plasmids

Plasmids constitute a significant proportion of the *Borrelia* genome both quantitatively and genetically. For example, 17% of the coding capacity of a low passage infectious isolate of *B. burgdorferi* B31 is in plasmid DNA (Marconi *et al.*, 1993a; Sadziene *et al.*, 1993b). Both linear and circular plasmids have been observed.

Linear plasmids. Linear *Borrelia* plasmids were first observed in *B. hermsii* (Plasterk *et al.*, 1985) and subsequently found to be a feature of all Lyme disease spirochaetes. Their role in antigenic variation has been the subject of a recent review (Barbour, 1993). In *B. hermsii* linear plasmids harbour the genes for the important antigens known as variable major proteins (Vmps) and provide the vehicle for the translocation of these genes that is responsible for antigenic variation (see section on antigenic differences). In Lyme disease spirochaetes, *ospAB* and *ospD*, which encode outer-surface proteins (Osps), are located on linear plasmids. Other linear-plasmid-encoded functions remain to be elucidated.

The linear *B. hermsii* plasmid bp7E, which carries the expressed version of the *vmp7* allele, has a copy number of 14 per cell (0·9 per chromosome) in cells grown in mice and one-quarter to one-half this number in broth-cultured organisms (Kitten & Barbour, 1992). The type strain *B. burgdorferi* B31 has four different linear plasmids (Fig. 1). The 16 and 49 kbp plasmids of *B. burgdorferi* B31 also have a copy number of approximately one per chromosome (Hinnebusch & Barbour, 1992). These copy number values for both *B. hermsii* and *B. burgdorferi* suggest that replication and partitioning of plasmids and chromosome are tightly coupled in these organisms. Such tight coupling may be achieved genetically and/or by the organization of the individual borrelial genomic elements (chromosome and plasmids) into nucleoids which are distributed along the length of the cell and which replicate and segregate as discrete units (Kitten & Barbour, 1992).

The telomeres of the linear plasmids. Two questions of major interest follow from the discovery of linear plasmids in *Borrelia*. What are the structures of the telomeres and how are they replicated? A definitive answer to the first question has been provided by the work of Barbour and his associates (Hinnebusch & Barbour, 1991). After initially establishing that the linear plasmids have covalently closed ends that are disrupted by S1 nuclease but not protease treatment, they isolated terminal fragments from the 16 kbp linear plasmid of *B. burgdorferi* B31, and directly determined their nucleotide sequences. Noteworthy was the discovery of a palindrome that constituted an inverted terminal repeat for the plasmid (Fig. 6). At each telomere the two strands of DNA are covalently connected by four, unpaired nucleotides that form a hairpin loop. Significant homology, indicative of a common evolutionary origin, exists between the nucleotide sequences at the left ends of the 16 kbp plasmid, the 49 kbp *B. burgdorferi* B31 linear plasmid, and a linear *B. hermsii* plasmid. Another prokaryote replicon, that of the lysogenic coliphage N15, exists as a linear double-stranded DNA molecule with palindromic terminal hairpin loops (Svarchevsky & Rybchin, 1984). However, the non-borrelial telomere which most clearly resembles the 16 kbp *B. burgdorferi* plasmid telomere is that of a eukaryotic virus, African swine fever virus. This close resemblance led Hinnebusch and Barbour to propose the intriguing possibility that the linear *Borrelia* plasmids arose by a horizontal genetic transfer between kingdoms (Hinnebusch & Barbour, 1991). The fact that African swine fever virus and *B. duttoni*, an aetiologic agent of

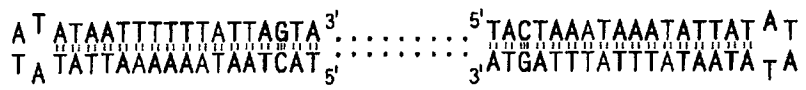

Fig. 6. Nucleotide sequences of the telomeres of the 16 kbp linear plasmid from *B. burgdorferi* B31. Nucleotides shown in bold type comprise an indirect repeat sequence. The data are from Hinnebusch & Barbour (1991).

relapsing fever, share the same tick host adds credence to this proposal.

Circular plasmids. Circular plasmids carried by bacterial pathogens frequently confer virulence properties and other desirable characteristics such as antibiotic resistance. The number of circular plasmids in *B. burgdorferi* varies between strains and within a given isolate since some are lost during *in vitro* cultivation. For example, the complement of circular plasmids in *B. burgdorferi* Sh-2-82 declined from six to four in the course of 2 years of *in vitro* passaging from its initial low-passage state (less than 10 generation times) (Simpson *et al.*, 1990a). The lost plasmids were found to be non-essential for infection of *Peromyscus leucopus*. In the Lyme disease spirochaetes, circular plasmids have been shown to carry the following loci: *ospC* (see section on OspC), homologues of *guaA* and *guaB*, which encode the enzymes that convert XMP to GMP and IMP to XMP, respectively (N. Margolis, D. Hogan & P. Rosa, unpublished), and a class of species-specific repetitive DNA found in a number of circular plasmids but nowhere else in the genome (Simpson *et al.*, 1990b). While it has been suggested that this repetitive DNA may encode functionally similar proteins such as related antigens, further work is needed before any firm conclusions about its function are reached.

Major outer-membrane proteins of *Borrelia*

Proteins of high abundance that are found in the outer membrane of borreliae have been studied extensively because they could be major antigens and play an important role in pathogenesis. In Lyme disease spirochaetes, the relevant proteins are referred to as OspA and OspB while in *B. hermsii* they are the Vmps. Despite considerable effort, the functions of OspA and OspB remain poorly defined, although a recent report (Sadziene *et al.*, 1993a) showed that a mutant strain of *B. burgdorferi* in which OspB was truncated and under-expressed had diminished penetration capability and infectivity. Importantly, this report emphasized the value of studying antibody-resistant mutants of infectious isolates as a means of determining the role of the Osps in pathogenesis.

The ospAB operon in Lyme disease spirochaetes

The determination that *ospA* and *ospB* are organized in an operon was made by deletion analysis and transposon Tn*5* mutagenesis of a plasmid which expressed both OspA and OspB in *E. coli* (Howe *et al.*, 1986). The insertion of Tn*5* within *ospA* abolished expression of *ospB*, suggesting transcription of both genes from a single promoter. This was confirmed by the isolation of a 2·2 kbp transcript that hybridized with *ospA*- and *ospB*-specific probes. In *B. burgdorferi*, *ospAB* is carried on a 49 kbp linear plasmid (Barbour & Garon, 1987) while in *B. garinii* and *B. afzelii* it is present on 55 and 56 kbp linear plasmids, respectively (Samuels *et al.*, 1993). Determination of the nucleotide sequence of *ospAB* from *B. burgdorferi* B31 was notable since it provided the first nucleotide sequence of borrelial DNA (Bergström *et al.*, 1989). The deduced translation products from *ospA* and *ospB* were 273 and 296 amino acids long, respectively, and contained signal sequences with sites (Leu-X-Y-Cys) for processing by signal peptidase II. A combination of results confirmed that OspA and OspB are lipoproteins (Brandt *et al.*, 1990). In particular, it was shown that fatty acids are covalently linked to these two peptides in a way typical of most bacterial lipoproteins.

OspA and OspB differ in both their molecular masses and the relative amounts of their expression (for a review see Wilske *et al.*, 1992). As early as 1983 a panel of monoclonal antibodies recognizing OspA and which discriminate between strains was developed (Barbour *et al.*, 1983). Within the *B. burgdorferi* species, OspA was found to be 99% identical in three different strains (Bergström *et al.*, 1989; Wallich *et al.*, 1989; Fikrig *et al.*, 1990). In contrast, there were significant inter-species differences with 73–86% identity for OspA throughout the Lyme disease spirochaetes (Eiffert *et al.*, 1992; Jonsson *et al.*, 1992; Zumstein *et al.*, 1992). A similar degree of divergence between the three species was seen in OspB (Jonsson *et al.*, 1992). The most highly conserved region of OspA is the NH_2-terminus. The two Osp proteins show 53% overall sequence identity, indicating a recent evolutionary duplication of an ancestral *osp* gene. It has been suggested that this duplication occurred before the geographical dispersion of the strains (Bergström *et al.*, 1989).

Antigenic differences or true antigenic variation?

Borrelia are effective at evading the host immune response. The mechanisms by which they do this are better understood for *B. hermsii*, where true antigenic variation is well-documented, than they are for the Lyme disease spirochaetes.

Infection of a human (or experimental animal model) by a relapsing fever spirochaete such as *B. hermsii* leads to a characteristic pattern of periodic fevers recurring at intervals of 4–7 d (for a review of antigenic variation in relapsing fever *Borrelia* species, see Barbour, 1990). Blood levels of borreliae rise and fall coincidentally with the fever. Analysis of this phenomenon was aided by the

observation that inoculation of a mouse with a single *B. hermsii* HS1 cell yielded progeny spirochaetes with 25 different serotypes. This experiment demonstrated significant capacity for antigenic variation in *B. hermsii*. The variation, which is called multiphasic antigenic variation, is reversible.

At the molecular level, serotype specificity in *B. hermsii* is determined by the abundant membrane lipoproteins referred to as Vmps, which vary from 21 to 39 kDa in molecular mass. The genes encoding these proteins are located on linear plasmids and are silent except when they are present in a site immediately adjacent to one of the linear plasmid telomeres. Molecular analysis of the *vmp* locus in the 25 *B. hermsii* HS1 serotypes referred to above indicated that the different *vmp* genes have related sequences (39–78%) and are flanked on each side by identical nucleotide sequences (Restrepo *et al.*, 1992). These latter sequences are presumed to act as sites for homologous, inter-plasmidic recombinations which enable different *vmp* alleles to be translocated between silent and active (or expression) sites, thereby bringing about a change in the nature of the expressed Vmp with consequent antigenic variation. The translocation to the expression site, which has a subtelomeric location, is predominantly unidirectional and is either a form of gene conversion or a double crossover in which the previously active allele is lost.

Burman *et al.* (1990) noted that of the known mechanisms used by pathogens to achieve antigenic variation, the one that most closely resembles the *B. hermsii* mechanism is that used by a eukaryote, the African trypanosome. In the trypanosome, DNA rearrangements are used to create a telomeric environment for the expression of hitherto silent genes which encode different forms of the abundant surface protein (Vsg). The secondary structures of Vmps and Vsgs are similar although there is no sequence similarity between the *vmp* and *vsg* genes (Burman *et al.*, 1990). In the absence of additional experimental data we can do no more at present than draw attention to this surprising similarity between these two phylogenetically quite distant organisms.

Following infection by Lyme disease spirochaetes, the pathogen evades the immune response and then establishes a chronic infection. One possibility is that this state is achieved through changes in the outer-surface proteins such as OspA, OspB and OspC, but current evidence for a mechanism involving true antigenic variation is not convincing. Different strategies have been used in an attempt to identify the mechanisms of cell-surface changes in *B. burgdorferi*. In one approach *B. burgdorferi* was examined to determine whether bona fide antigenic variation in OspB had occurred. The progeny of single organisms grown *in vitro* were examined using several clones obtained from HB19, a human isolate (Bundoc & Barbour, 1989). Variants in which OspB differed in molecular mass and its reactivity with monoclonal antibodies were found, but none of the variations were caused by major DNA rearrangements or the non-transcription of *ospB*.

In a second approach, escape variants were selected by obtaining growth of *B. burgdorferi* in the presence of monoclonal antibodies directed against OspA or OspB or both (Sadziene *et al.*, 1992, 1993a; Coleman *et al.*, 1994). Three kinds of variants were obtained. The first had OspA and OspB proteins of the same electrophoretic mobilities as the wild-type but were no longer recognized by selecting antibodies. The second had truncated derivatives of either OspA, OspB or both. The third expressed neither OspA nor OspB and had lost the 49 kbp linear plasmid carrying *ospAB*. Two major conclusions concerning *B. burgdorferi* arose from these studies: (a) OspA and OspB are not essential for *in vitro* growth and (b) reduced size and expression of OspB are associated with lower virulence. A third analysis by Rosa *et al.* (1992) yielded data suggesting that recombination between *ospA* and *ospB* could be a means of generating antigenic diversity.

In conclusion, despite concentrated efforts directed towards the analysis of variability in the major outer-surface proteins of Lyme disease spirochaetes, a mechanism of antigenic variation similar to that found in *B. hermsii* has not been demonstrated.

OspA and OspB recombinant proteins are being evaluated for the development of a vaccine against Lyme disease. Immunization of mice was obtained after syringe injection or infected tick bites (Fikrig *et al.*, 1990, 1992). The mode of immunization is relevant since the *Borrelia* load delivered through a syringe can be up to 10^8 organisms whereas tick bites deliver at most 10^6 (Gern *et al.*, 1993). In addition, tick saliva possesses antiinflammatory properties which have been shown to lower host immune defences. The only vaccine commercialized in the USA is for dogs. This procedure has a 40% efficiency (for a review, see Barbour & Fish, 1993). It is not yet clear whether a human vaccine will provide a realistic way of combating Lyme disease.

OspC, a member of the Vmp family

No function had been ascribed to a circular *Borrelia* plasmid until recently when *ospC* was localized to the 27 kbp plasmid of Lyme disease spirochaetes (Marconi *et al.*, 1993a; Sadziene *et al.*, 1993b). *ospC* encodes the immunodominant major protein formerly known as pC (Fuchs *et al.*, 1992), an outer-membrane protein, which exhibits 70–74% identity across the three species of Lyme disease spirochaetes (Jauris-Heipke *et al.*, 1993; Wilske *et al.*, 1993). Interestingly, all the other analysed *Borrelia* species carry *ospC* on one or more linear plasmids (Marconi *et al.*, 1993b). OspC belongs to a family of surface-exposed proteins which comprise the Vmp of *B. hermsii* (Carter *et al.*, 1994) and has been proposed as a candidate for a vaccine against Lyme disease (Preac-Mursic *et al.*, 1992).

The 27 kbp plasmid of Lyme disease spirochaetes is either very stable or essential for *in vitro* growth because it has never been cured. Sadziene *et al.* (1993b) analysed derivatives of the *B. burgdorferi* B31 lineage for plasmid

content and the expression of *ospC* and found that although *ospC* was present throughout, some derivatives did not produce OspC. In particular, OspC was produced by two high-passage isolates, *B. burgdorferi* B312 and B314, but not by the parental strain, B31, or another high-passage derivative, B313. The plasmid complement of these four strains differed in a way that suggested that a product of the 16 kbp linear plasmid, lp16, specifically caused negative regulation of *ospC*. This hypothesis remains to be tested, since the obvious way of achieving it, by introducing lp16 back into strain B314, is not possible until genetic transfer is available for *B. burgdorferi*.

The regulation of *ospC* expression appears to be interdependent with that of *ospAB*, since low-passaged strain CA-11 2A does not express *ospAB*, but expresses *ospC* (Wilske *et al.*, 1993). After 40 passages, the situation was reversed (Margolis & Rosa, 1993). There is a clear need for further analyses of this area to determine if a regulatory circuit exists that controls the levels of the various Osps.

OspD, a putative virulence factor

An interesting search for proteins important for pathogenesis and immunogenicity was undertaken by Norris *et al.* (1992) using the rationale that proteins present in a low-passage isolate and absent from the corresponding high-passage derivative are potential candidates. Two-dimensional gel electrophoresis was used to identify four proteins associated exclusively with low-passage isolates. The proteins were isolated and found to be lipoproteins. The gene for the most abundant, OspD, a 28 kDa protein, was cloned and located on a 38 kbp linear plasmid. A similar gene is present in plasmids in the 35–40 kbp range in other low-passage strains from *B. burgdorferi* and *B. garinii*. The predicted amino acid sequence of OspD had no significant similarity with the sequences of any proteins in the databases. An intriguing glimpse into the regulation of *ospD* was provided by the discovery of seven 17 bp direct repeats, each containing a −35 and a −10 element of a σ^{70}-like promoter sequence, in its 5′ flanking DNA. Transcription initiation was mapped to the closest promoter to the start codon. It was proposed that the six upstream promoter sequences could serve as stacking sites for RNA polymerase molecules awaiting a free promoter and/or sites which undergo recombinational events affecting promoter function. The OspD protein was expressed by low-passage strains but its presence is not required for infectivity. It therefore cannot be a virulence factor.

Endoflagella, characteristic of spirochaetes

Spirochaetes have endoflagella, which are the functional equivalents of the external flagella found in other bacteria. A unique feature of spirochaetal endoflagella is that they are contained within the periplasm (for a review see Charon *et al.*, 1992). One or several flagella, depending on the spirochaete genus, are inserted subterminally at each end of the cell cylinder and provide an efficient motility system for the organism.

In contrast to most spirochaetal endoflagellar filaments, which are composed of a number of polypeptides, the *B. burgdorferi* endoflagellum consists of only one type of subunit, the 41 kDa protein Fla. Analysis of *fla* predicts that the protein is comprised of 336 amino acids and has a molecular mass of 35·8 kDa. The discrepancy between the measured and predicted molecular masses of Fla may result from glycosylation of the gene product (Luft *et al.*, 1989). The sequence of Fla is highly conserved (96–97% identity) within the Lyme disease spirochaetes (Wallich *et al.*, 1990; Gassmann *et al.*, 1991; Jauris-Heipke *et al.*, 1993) and also has a significant degree of homology with the analogous flagellin protein in other spirochaetes (*B. hermsii* and *T. pallidum*) and in phylogenetically distant bacteria (*Bacillus subtilis*, *Serratia marcescens*, *E. coli* and *Salmonella typhimurium*).

A flagella-less *B. burgdorferi* mutant, characterized by a reduced ability to penetrate human endothelial cell layers, has been reported (Sadziene *et al.*, 1991). The mutation involved neither a major DNA rearrangement nor the failure of transcription and was therefore probably a nonsense or frameshift mutation.

Recently, two other flagellin genes, *fliG*, encoding flagellar shift protein, and *flgE*, encoding the hook protein, have also been isolated (N. Charon, unpublished results). The three flagellin genes are not arranged in a single operon but are scattered on the chromosome (Fig. 3).

Potential involvement of heat-shock proteins in autoimmune reactions

It has been suggested that many late symptoms of Lyme borreliosis are caused by autoimmune reactions resulting from structural similarities between borrelial antigens and host proteins. Heat-shock proteins (Hsps), which are highly conserved and present in all cellular organisms (Lindquist, 1986), may be important in the development of these reactions through molecular mimicry by the pathogen. In this regard, it is of interest that a monoclonal antibody specific for the *B. burgdorferi* flagellin detects the human chaperonin HSP60 (Dai *et al.*, 1993). *Borrelia* must survive in the two different environments of an arthropod vector at ambient temperature and a warm-blooded host. The transmission from vector to host by the bite of a tick will cause a sudden increase in temperature similar to the heat stress normally responsible for the heat-shock response. For these two reasons, the molecular biology of borrelial Hsps has attracted attention (Carreiro *et al.*, 1990; Cluss & Boothby, 1990).

The first Hsp that was characterized at the molecular level was the so-called 'common antigen' (Hansen *et al.*, 1988), which is homologous to GroEL from other bacteria. Subsequently, *dnaK*, *dnaJ* and *grpE* were isolated and analysed (Anzola *et al.*, 1992a, b; Tilly *et al.*, 1993). *B. burgdorferi* DnaJ contains four cysteine-rich repeats, each with the Cys-X-X-Cys-X-Gly-X-Gly motif characteristic of DnaJ proteins. *B. burgdorferi dnaJ* and *grpE* complement the corresponding *E. coli* mutations while *B. burgdorferi dnaK* does not complement *E. coli dnaK* mutations. The

grpE, *dnaK* and *dnaJ* genes may form an operon with the promoter upstream of *grpE*.

Genetic transfer and genetic tools

As mentioned in the first section, the genetic analysis of *B. burgdorferi* has been hampered by the absence of any exchange mechanism, such as transduction or transformation, that allows the introduction of genes into *Borrelia* cells. Bacteriophage particles have been visualized by electron microscopy in the culture of a Lyme disease spirochaete but have not been physically isolated (Hayes *et al.*, 1983; Neubert *et al.*, 1993). As yet, none of the linear and circular plasmids have been developed as a tool for gene delivery. Electroporation has resulted in homologous recombination (D. S. Samuels, personal communication) within *Borrelia* wild-type cells transformed with a linear fragment of *Borrelia* DNA carrying a mutation in *gyrB* (Samuels *et al.*, 1994).

Undoubtedly, the development of a transformation system for *Borrelia* is essential for sophisticated genetic analysis in this genus and would be much welcomed by investigators in the field. Similarly, a method for conjugal transfer between a well-studied bacterium such as *E. coli* or *B. subtilis* and *Borrelia* would provide major opportunities for studying the pathogenic and antigenic properties of these spirochaetes.

For editorial reasons, we have carefully limited the number of references cited, using reviews which can lead interested readers back to the original paper wherever possible. We hope that our colleagues will excuse us for not citing all their papers and understand the dilemma we faced.

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